MOLECULAR CLONING, CHARACTERIZATION, AND EXPRESSION OF MULTIPLE PORCINE CYTOCHROME P450 AROMATASE COMPLEMENTARY DEOXYRIBONUCLEIC ACIDS AND CHROMOSOMAL GENES

By

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To my parents who have educated me to be the person I am

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Abstract of Dissertation Presented to the Graduate School
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In a number of mammals, the periimplantation embryo secretes estrogens which may act to modify the uterine microenvironment for implantation. Pig blastocysts transiently synthesize and secrete large amounts of estrogens during periimplantation. However, there is little information concerning the developmental regulation of embryonic aromatase gene expression.

To address this, the expression pattern of this gene in pig embryos was investigated and the transiently high expression of aromatase mRNA, coincident with the rapidly changing concentrations of IGFs within the uterine microenvironment, was found. Porcine aromatase cDNAs encoding an aromatase protein of 503 amino acids were isolated from Day 12 embryos by molecular cloning and were sequenced. It was demonstrated that the aromatase transcript (33F clone-type) containing a unique 5' untranslated exon (E1A) was the major

mRNA form expressed in periimplantation embryos. No significant homology was found between the E1A sequence and 5' untranslated exons of aromatase mRNAs identified in other species including that for equine aromatase cloned from Day 14 embryos. Expression of aromatase transcripts in pig endometrium as well as placental tissues during pregnancy was demonstrated. Two additional aromatase isoforms were identified from embryos (A10 clone) and placenta (P2 clone), respectively. The A10 clone encoded a 354 amino acid, aromatase-related protein due to deletion of internal exons 4-6 by an alternative RNA splicing mechanism. The P2 cDNA clone differed from the 33F cDNA clone in seventy-seven nucleotides. The P2 clone-type transcript was found to be the major aromatase mRNA form expressed in endometrium and placenta during midpregnancy. Genomic DNA fragments encoding different isoforms of porcine aromatase were obtained from a genomic DNA library and by genomic PCR cloning and their respective arrangements of exons and exon-intron boundary sequences were determined. It was demonstrated that multiple isoforms of porcine aromatase originate from multiple aromatase chromosomal genes and potentially from RNA editing mechanisms.

A unique gene of approximately 30 kb in size and encoding the two untranslated exons (E1A and E1B) as well as nine coding exons is likely responsible for the major embryo aromatase transcript, transiently expressed in pig blastocysts during the periimplantation period. The protein product of this gene may be involved in the synthesis of unknown phenolic compounds and estrogens implicated in embryo-maternal communication during periimplantation.

CHAPTER 1
INTRODUCTION

It has been known for some time that blastocysts of several mammalian species including the rat, hamster, rabbit, pig, horse, donkey, camel, and human secrete estrogens at defined early developmental stages. Although the role(s) of these estrogens is for the most part unclear, evidence suggests that these hormones may be involved in the initiation of uterine changes associated with implantation. The amount of estrogens secreted by porcine embryos during the periimplantation period (Days 11&12) is extremely high among the species identified above. The biological functions of estrogens secreted by porcine embryos during the periimplantation period have been studied with a particular focus on maintenance of pregnancy via prevention of luteolysis of the corpus luteum. In addition, we and others have speculated that other functions of estrogens secreted by porcine embryos might be to serve as regulators of maternal uterine gene expression and protein synthesis and secretion, which in turn are essential for embryonic growth and development and implantation.

Cytochrome P450 aromatase, the product of the CYP19 gene, is the microsomal steroidogenic enzyme that converts androgens (C_{19}) into estrogens (C_{18}) and is composed of two subunits, cytochrome P450 aromatase and NADPH-cytochrome P450 reductase. The name "aromatase" was derived from its catalytic activity of aromatization of the A ring of androgens after three successive hydroxylation reactions using 3 molecules of O_2 and 3

molecules of NADPH in the reaction. The NADPH-cytochrome P450 reductase is ubiquitous in steroidogenic tissues, so the tissue specificity of estrogen biosynthesis is determined by the expression of the cytochrome P450 aromatase subunit. The human is the only species for which the detailed gene structure and the molecular mechanisms of aromatase gene expression have been intensively investigated. The size of the aromatase genomic DNA in the human is much longer (at least 80 kb) than that for other cytochrome P450 genes and exons 2 through exon 10 of this gene encode the amino acids of aromatase. Several 5' untranslated exons located upstream of exon 2, generated via alternative promoter usage and exon splicing mechanisms, are tissue-specifically expressed to produce different mRNA species.

There has accumulated a substantial body of biochemical and immunohistochemical data demonstrating the developmental regulation of steroidogenic enzyme activity in periimplantation embryos, especially those of the pig. Estrone (E1) is known to be a major estrogen synthesized by periimplantation porcine embryos. In addition, there exist other unknown phenolic compounds produced by these embryos whose biological functions have yet to be investigated. Porcine blastocysts also synthesize and secrete large amounts of catechol estrogens (E_2-2/4-OH) and 15α-hydroxy-estradiol-17β (15α-OH-E_2) transiently during the periimplantation period and in very similar temporal fashions as for estrogens. The recent identification of cytochrome P450 aromatase as the human placental estrogen-2/4-hydroxylase suggests the possibility that catechol estrogens may be steroidogenic products of the porcine blastocyst aromatase complex as well.

There is little information concerning the nature of the regulation of steroidogenic

enzyme genes utilized in the blastocyst. I have hypothesized that there may be a embryo-specific transcription factor(s), which interacting with a specific DNA sequence upstream of an embryonic aromatase promoter, is responsible for the unique temporal patterns of blastocyst estrogen production. Therefore, to begin to elucidate the molecular mechanism(s) underlying the temporal expression of the aromatase gene in embryos using the pig as animal model, the present studies were conducted to: 1) investigate the expression pattern of the aromatase gene in pig blastocysts during the periimplantation period, 2) clone the complementary deoxyribonucleic acid(s) of aromatase that is expressed in pig embryos and whose product is responsible for the synthesis of estrogens and perhaps other yet unknown estrogenic derivatives and, 3) clone and characterize the chromosomal DNA encoding embryonic aromatase and the highly related aromatase gene expressed in the endometrium and placenta.

CHAPTER 2
LITERATURE REVIEW

Functions of Blastocyst-derived Estrogens during the Periimplantation Period

The term "maternal recognition of pregnancy" describes a process by which the blastocyst (conceptus) acts to prevent regression of the corpus luteum (luteolysis) and thereby maintains luteal secretion of progesterone necessary for endometrial function during pregnancy (Knobil et al., 1988). Prostaglandin $F_{2\alpha}$ is a common luteolytic agent (luteolysin) in many species. Different species use different types of embryonic signals to inhibit the synthesis or antagonize the action of the luteolysin (Knobil et al., 1988; Bazer, 1992; Thatcher et al., 1995). Continuous secretion of progesterone by the corpus luteum is the hormonal support for uterine differentiation and endometrial secretion, which helps to provide a favorable environment for embryonic growth, development, and implantation. The pig is the only known species in which blastocyst-elicited estrogen is the signal for maternal recognition of pregnancy.

maternal recognition of pregnancy: Subcutaneous injection of estrogens on Day 11 of the estrous cycle maintained functional corpora lutea in gilts (Gardner et al., 1963). The absence of embryos in the uterine horn between Days 14 and 16 led to bilateral regression of corpora lutea (CL) (Dhindsa and Dziuk, 1968) and more than four embryos (two embryos for each horn) were required to maintain pregnancy (Polge et al., 1966). The presence of

prostaglandin F (PGF) in blood from the utero-ovarian vein during the luteal phase of the oestrous cycle was demonstrated (Gleeson et al., 1974). Injection of $PGF_{2\alpha}$ into bilaterally hysterectomized gilts resulted in CL regression and a decrease in serum progesterone, suggesting that $PGF_{2\alpha}$ is a luteolysin in pigs (Moeljono et al., 1976). Moreover, it was hypothesized that the estrogenic activity of porcine blastocysts during Days 10 to 12 of pregnancy was involved in the process of maternal recognition of pregnancy in this species (Perry et al., 1973; Perry et al., 1976). High amounts of PGF secretion by endometrium during the luteal phase of the estrous cycle was demonstrated using primary cultures of pig endometrium (Patek and Watson, 1976). Utero-ovarian $PGF_{2\alpha}$ concentrations were lower in gilts treated with estradiol valerate on Day 9 after onset of estrus than in control animals, again indicating that estrogen plays a role in preventing $PGF_{2\alpha}$ release from the uterus (Frank et al., 1977). Further studies demonstrated that estrogen treatment increases the uterine $PGF_{2\alpha}$ level (Frank et al., 1978) and that PGF concentrations in uterine luminal fluids were greater in pregnant than nonpregnant gilts (Zavy et al., 1980). These results suggested that embryonic estrogen does not actually reduce the synthesis of PGF but instead changes the direction of PGF secretion from endometrium to the uterine lumen rather than to the circulation (Bazer and Thatcher, 1977). It was suggested that two periods of estrogen injections (first period, Day 11 and second period, Day 14) were required to maintain corpora lutea beyond Day 60 (Geisert et al., 1987). The subsequent findings of temporal increases of calcium ion content in uterine flushings during the time pig blastocysts are elongating (Days 11 and 12) (Geisert et al., 1982a) and increases in Ca content after treatment with estrogen (Geisert et al., 1982b) suggested that blastocyst estrogens stimulate

intraluminal release of calcium from the uterine epithelium, which in turn is associated with increased intraluminal prostaglandin secretion. Perfusion with calcium inophore, a stimulator of intracellular calcium secretion, altered the direction of endometrial PGF secretion from the endocrine to uterine luminal direction (exocrine); neither estrogen nor prolactin alone altered the direction of PGF secretion, but the combination of estrogen and prolactin led to reorientation of PGF secretion (Gross et al., 1990). These results confirmed the involvement of calcium in the reorientation of PGF secretion and implied an interaction of estrogen and prolactin in these events. However, exactly how calcium, estrogen and prolactin are involved in the reorientation of PGF secretion remains unclear.

Uterine arterial blood flow increases from Day 11 to 13 of pregnancy as compared with the corresponding days of the estrous cycle and then declines by Day 15 (Ford et al., 1982a) and intrauterine injections of estradiol-17β (E_2) on Day 11 increased uterine blood flow eight to ten-fold within 12 h and remained elevated through to Day 17, whereas uterine blood flow (UBF) of control animal remained unchanged (Ford et al., 1982b). Thus, it is likely that elevated uterine blood flow due to the action of embryonic estrogens serves to dilute the effective concentration of PGF, which may decrease the activity of PGF as the luteolysin. While the studies described above suggest that embryo-derived estrogens indirectly prevent luteolysis of corpus luteum via alteration of secretion of luteolysin, other studies indicated a direct luteotrophic role for blastocyst estrogens to maintain pregnancy (Garverick et al., 1982). Subcutaneous injections of estrogen on Day 12 of the estrous cycle delayed luteal regression and the usual decline in LH receptor levels, suggesting that E_2 affected luteal LH receptor levels via a mechanism independent of the uterus (Garverick et al., 1982).

embryo survival: Dickmann et al. (1976a) suggested that molula stage embryos of laboratory animals synthesize steroid hormones, which are essential for normal embryo development, at certain rates and in certain proportions . During the preimplantation period, pig embryos move within the two uterine horns (Dziuk et al., 1964), undergo remarkable morphological changes, and become regularly spaced with no overlap of tubular membranes from adjacent embryos within the uterine horn (Perry and Rowland, 1962; Anderson, 1978). Pig blastocysts grow at a rate of 0.25 mm/hr at early developmental stages (spherical forms; 4-9 mm in diameter) and then elongate at a rate of 30 to 45 mm/h to become the filamentous forms (>100 mm), via an intermediate stage (tubular forms) (Geisert et al., 1982b). A large amount of embryo loss occurs during this period in the pig (Perry and Rowland, 1962; Anderson, 1978).

Experiments have indicated that estrogens and histamines are involved in the intrauterine migration of porcine embryos during the preimplantation period (Pope et al., 1982). Gilts treated with exogenous estradiol-17β during Days 12 and 13 of pregnancy have less embryo loss than do animals treated on Days 9-10, suggesting that the uterine environment on Days 12 and 13 is more tolerant of exogenous treatment with estrogen than that on Days 9 and 10 (Pope et al., 1986). Use of embryo transfer techniques led to the observation that synchronization of uterine environment and embryo development is necessary for optimal rates of embryo survival (Morgan et al., 1987; Geisert et al., 1991). Blair et al. (1991) demonstrated that premature exposure of the uterus to estrogen alters the uterine epithelial surface, resulting in failure of conceptus as to attach and subsequent embryonic mortality.

Bazer et al. (1988) reported that blastocysts of Meishan gilts develop more rapidly and more uniformly than do blastocysts of Large White gilts between Days 8 and 14 of gestation, which might result in higher embryo survival (Bazer et al., 1988). It was also suggested that early embryonic mortality in pigs is highly correlated with events of oocyte and follicular maturation, since oocytes of later-ovulating follicles were progenitors of smaller embryos within a litter, which probably were at a disadvantage over the more advanced littermates on Day 12 (Pope et al., 1990; Xie et al., 1990). Uniformity in embryo development may therefore, be advantageous for embryo survival. On the other hand, Wilmut et al. (1992) found no significant developmental differences between embryos of the Meishan and the Large White breeds, suggesting that the greater uniformity of Meishan embryo development within a litter is the major determinant for their greater embryo survival. The finding of a relatively short time range (1.8 h) of ovulation in the pig indicates no relationship between time of ovulation and conceptus diversity (Soede et al., 1992). No significant difference in sex ratio of Day 10 pig embryos was found, although female embryos are smaller (i.e., less developed) than male embryos, indicating that early embryo survival is neither embryo size nor embryo sex-dependent (Cassar et al., 1994).

It is unclear whether embryo-derived estrogens directly affect pig embryo growth, development, or survival since there is no evidence for the presence of estrogen receptors in pig blastocysts, although estrogen receptor mRNA was observed indeveloping mouse blastocysts (Hou and Gorski, 1993).

uterine biology: Uterine secretions (histotroph) provide nutrients for embryos and a favorable environment for development and are especially important for species characterized

by the non-invasive, epitheliochorial type of placentation, such as pigs, sheep, goats, cows, and horses (Roberts and Bazer, 1988; Simmen and Simmen, 1990). The pig uterus elongates throughout the first 18 days of pregnancy, and this elongation is most rapid between the 2nd and 6th days when a 50% increase in length occurrs (Perry and Rowlands, 1962). Uterine capacity is unrelated to the number of embryos surviving during the first 18 days after mating (Anderson, 1978). A temporal correlation of uterine secretory content of calcium, total proteins, and PGF and PGE with estrogen on Days 11 and 12 of pregnancy was noted (Geisert et al., 1982a). Administration of estradiol valerate to gilts on Days 11 through 15 of the estrous cycle resulted in an induction of total protein and calcium in the uterine luminal fluid (ULF) at 12-24 hours post-treatment (Geisert et al., 1982c). The increase in calcium preceded the increase in protein, implicating the involvement of calcium in E_2-stimulated endometrial epithelial secretory vesicle exocytosis (Young et al., 1987). The endometrial secretory response to estrogen occurs after Day 10 of the estrous cycle (Geisert et al., 1987).

Temporal increases in ULF IGF-I content (Simmen et al., 1988; Letcher et al., 1989) and endometrial IGF-mRNA abundance (Simmen et al., 1992) were found on Days 10-12 of pregnancy in pigs. IGF-I mRNA abundance also peaks at Day 12 of the estrous cycle, although levels of IGF-I are higher at Day 12 of pregnancy compared to corresponding day of cycle (Simmen et al., 1992). Exogenous treatment with estrogen (E_2) or progesterone (P_4) increased uterine IGF-I mRNA levels and ULF IGF-I protein content in gilts (Simmen et al., 1990). These results indicated that the transient secretion of estrogens might be involved in the regulation of endometrial IGF-I aromatase expression and/or secretion. An increase in uterine IGF-I mRNA abundance with estrogen treatment was also found for

ovariectomized rats (Murphy et al., 1987).

The presence of IGF-I receptors (Corps et al., 1990) and corresponding mRNA (Green et al., 1995) in pig blastocysts during early pregnancy has been demonstrated. IGF-I selectively stimulated protein synthesis in the embryonic disc region of pig blastocysts (Estrada et al., 1991). Estrogens also induce the production of other uterine growth factors (Gardner et al., 1989; Huet-Hudson et al., 1990) and proto-oncogenes (Stancel et al., 1993; Weisz and Bresciani, 1993) in the rat and other mammalian species. However, the role of estrogens in expression of growth factors, their receptors, and proto-oncogenes in the blastocyst (Whyte and Stewart, 1989; Vaughan et al., 1992; Zhang et al., 1992; Simmen and Simmen, 1991; Adamson, 1993) has not been explored.

implantation: The porcine placenta is a classic example of the diffuse, epitheliochorial type of placentation (Perry, 1981; King et al., 1982). Placentation initiates around Days 13 and 14 of pregnancy, when chorionic caps enclose the protruding epithelial proliferations of endometrium to immobilize the blastocysts (Danzer, 1985) Exogenous treatment with estrogen and progesterone during the periimplantation period results in larger placentae by Day 50 of gestation and greater uterine growth as well (Dalton and Knight, 1983). The noted morphological changes and increased vascular permeability of the endometrium coincident with elongation of pig embryos (Keys et al., 1986; Keys and King, 1988) suggested the probable involvement of embryonic estrogens. Early exposure of pregnant gilts to estrogens (days 9 and 10) resulted in loss of the uterine glycocalyx, which may be important for implantation, beginning on Day 14 (Blair et al., 1991). Thus, embryo mortality resulting from premature exposure to estrogens may be related to unfavorable

changes of the uterine endometrial surface during the period of conceptus attachment to endometrium. Both intrauterine and systemic treatment with estrogens on Days 11-15 of the estrous cycle induced folding along the mesometrial region of the uterus, similar to that occurring during the corresponding days of pregnancy (Keys and King, 1992).

Embryo-synthesized steroids are known to be important for implantation in laboratory animals (Dickmann et al., 1976a). Dickmann et al (1976b) suggested that rat blastocyst-derived estrogen is the causative factor for the local increases in endometrial capillary permeability noted during early pregnancy. Blastocyst development and implantation in rabbits was prevented by intraluminal treatment with anti-estrogens on Day 5 of pregnancy (Bhatt and Bullock, 1974; Dey et al., 1976). It was demonstrated that a single injection of estradiol was partially effective in sensitizing the uterus to a decidual stimulus in progesterone-treated mice, whereas two injections were fully effective (Martin, 1977). Ward et al. (1987) obtained evidence that estrogens diffusing from the blastocyst partially saturate the hormone binding capacity of the adjacent endometrium in the rat during implantation (Ward et al., 1978). However, successful implantation occurs for homozygous estrogen receptor null mutant mouse embryos (Korach, 1994), thereby calling into question a direct role for embryo-derived estrogens in the blastocysts.

Prostaglandins are likely involved in initiation of implantation via an increase in capillary permeability (Kennedy, 1977). Catechol estrogens (2-OH E_2) stimulated prostaglandin production by a homogenate of rat uterus (Kelly and Abel, 1980) and another study implicated catechol estrogens in the implantation process for the rat embryo (Dey et al., 1986). Catechol estrogens, but not estrogens, stimulated in vitro PG production by

both the preimplantation rabbit blastocyst and the endometrium (Pakrasi and Dey, 1983; Chakraborty et al., 1990). A single injection of either 3 ng oestradiol-17β or 50 ng of 4-OH-E_2 consistently induced implantation in mice (Hoversland et al., 1982). Pig blastocysts have catechol estrogen synthetase activity with a peak on Day 12 of pregnancy (Mondschein et al., 1985). However, there is no data available to date regarding the role of catechol estrogens in pig periimplantation conceptus development.

Biochemical Properties of Cytochrome P450 Aromatase

Cytochrome P450 is one member of the large family of oxidoreductase enzymes containing a single heme group. The name P450 (pigment 450) was derived from the distinct absorbance peak from 420 to 450 nm upon reduction with carbon monoxide (CO). The active site of cytochromes P450 binds the substrate and O_2 and reduces O_2 with electrons obtained from NADPH. Because both carbon monoxide and oxygen can bind to the same active site of the enzyme, carbon monoxide can competitively inhibit enzyme activity. Cytochrome P450 enzymes are involved in metabolism of steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, drugs, environmental pollutants, natural plant products, and alcohols. All cytochrome P450 are known to be the " b" type cytochrome and two hundred twenty one P450 genes and twelve putative pseudogenes have been found and characterized in diverse microorganisms and vertebrates (Nebert and Gonzalez, 1987; Nelson et al., 1993). Cytochrome P450 and other steroid oxidoreductase enzymes located in mitochondria and/or endoplasmic reticulum are responsible for synthesis of steroid hormones. Adrenal gland (especially the adrenal cortex), ovary, and testis are the major organs for the synthesis of

steroid hormones in mammalian species (Miller, 1988; Hanukoglu, 1992).

biochemical mechanisms of aromatization: It was first shown that human placental tissues could convert androstenedione to estrone via the intermediates, 19-hydroxyandrostenedione and 19-oxoandrostenedione (Meyer, 1955; Ryan, 1959; Morato et al., 1961). Thompson and Siiteri (1974a) reported that the addition of androstenedione and NADPH to human placental microsomes resulted in estrogen formation with concomitant formation of H_2O and formic acid byproducts. Three moles of O_2 and three moles of NADPH were required for one mole of estrogen formed via three hydroxylation steps (androstenedione → 19-hydroxyandrostenedione → 19-oxoandrostenedione → estrone). Enzyme kinetic and inhibition studies suggested that all three hydroxylation steps were carried out at the same active site of a single species of cytochrome P450 (Thompson and Siiteri, 1974b; Kelly et al., 1977). However, the extremely rapid conversion of 2β-hydroxy-derivatives from the incubation of $^{3}H/^{14}C$-radiolabeled androstenedione and the inequality in inhibition of the last hydroxylation step compared with the first two reactions was demonstrated by Goto and Fishman (1977). This result suggested that the last hydroxylation occurred at the 2β position of androstenedione in an additional active site of the enzyme, which differed from the first two C_{19} hydroxylation reactions mediated by a common active site (Goto and Fishman, 1977; Goto and Fishman, 1981; Fishman and Raju, 1981). In addition, inhibition of three hydroxylation steps by a cytochrome P450 reductase antibody provided evidence for the involvement of this cytochrome P450 in all three hydroxylation reactions of aromatization (Thompson and Siiteri, 1974b). Later, another model for the last hydroxylation step, suggesting that attack of 19-oxo group by heme ferric peroxide is

responsible for aromatic ring formation of estrone, was proposed (Cole and Robinson, 1988; Akhtar et al., 1982) and is supported by recent theoretical and empirical data (Akhtar et al., 1993; Korzekwa et al., 1993). Thus, the actual nature of the third hydroxylation step catalyzed by cytochrome P450 aromatase remains to be determined.

The human placental aromatase protein expressed in COS-1 monkey kidney tumor cells exhibited the catalytic activity of aromatization for three different substrates; androstenedione, testosterone, and 16α-hydroxyandrostenedione and was inhibited by the known aromatase inhibitors, 4-hydroxyandrostenedione and econazole (Corbin et al., 1988). This indicated that the three hydroxylation steps are catalyzed by a single aromatase enzyme which can utilize different substrates for aromatization, and this finding suppressed the previous controversies concerning the presence of multiple aromatase isozymes. In contrast, there is a considerable accumulation of the two intermediates for aromatization and subsequent delay in formation of estrogen and water by rat ovarian aromatase but not by human placental aromatase (Swinney et al, 1993). This result may be explained by tissue- and/or species-specificity of aromatase enzyme affinity for these intermediates and may be also indicative of the presence of multiple isoforms of aromatase, which have different activity for different intermediates or substrates. The finding of isoforms of porcine aromatase in this study and by others (Corbin et al., 1995) and the tissue-specific expression of these isoforms provides additional support for the latter idea.

purification of aromatase protein: Aromatase proteins with different purities and different specific activities were partially purified by several groups (Mendelson et al., 1985; Nakajin et al., 1986; Chen et al., 1986; Hagerman et al., 1986; Kellis and Vickery, 1987;

Harada et al., 1988; Yoshida et al., 1991). Molecular weight was estimated to be about 55,000 and the Km value of each purified aromatase preparation was quite variable, probably due to different degrees of purity of the enzyme. The aromatase purified by Nakajin et al. (1986) was extremely unstable and spontaneously formed P420 with a half-life of 2.5 days, whereas the half-life of the aromatase purified by Yoshida et al. (1991) was reported to be over 4 years upon storage at -80 C. It should be noted that human placenta is the only tissue used for the purification of aromatase to date and thus the possibility of tissue-specific isoforms of human aromatase cannot be ruled out. The purification of aromatase protein allowed researchers to raise antibodies to this protein. The N-terminal amino acid sequence from position 1 to 21 of the human placental aromatase was first determined by Chen et al. (1986), which in turn enabled this group to synthesize an aromatase oligonucleotide probe for screening of a placental library to isolate aromatase cDNA clones.

in vitro expression and mutagenesis studies of aromatase: The human placental aromatase protein was first expressed in COS-1 monkey kidney tumor cells and this protein catalyzed the aromatization of three different substrates, androstenedione (50 nM, Vmax 102), testosterone (55 nM, Vmax 148), and 16α-hydoxyandrostenedione (99 nM, Vmax 11) (Corbin et al., 1988). A Michaels-Menken constant (Km) of 34 nM and a maximum velocity of 23 pmol for the [1β,2β-^{3}H]androst-4-ene-3,17-dione were determined for human placental aromatase expressed in yeast *(Saccharomyces cervisiae)* (Pompon et al. 1989). A stable expression system of human placental aromatase using breast cancer cells (MCF-7), noncancerous breast cells (HBL-100), and Chinese hamster ovary cells also was developed (Zhou et al., 1990).

A mutagenesis study suggested that the N-terminal amino acids between residues 10 and 20 of human placental aromatase are important for microsomal targeting of the protein and/- or for conformational integrity (Amarneh et al., 1993). The carboxyl group in Glu^{302}, which is located in the I-helix (see Figure 4-3) responsible for substrate binding, is essential for the activity of cytochrome P450 aromatase (Graham-Lorence et al., 1991). An alteration of Pro^{308} to Phe^{308} (Zhou et al., 1991) or Pro^{308} to Val^{308} (Graham-Lorence et al., 1991) led to reduced enzyme catalytic activity for androstenedione, indicating that Pro^{308} may be involved in the bending of the I-helix of aromatase which is known to be critical for forming the substrate-binding pocket. Zhou et al. (1992) demonstrated that Asp^{309} in the I-helix was important for enzyme catalysis. Amino acid Thr^{310} in the I-helix is believed to bind to molecular oxygen and activate this oxygen and mutation of Thr^{310} to Ala^{310} inactivates the enzyme (Amarneh et al., 1993). An amino acid change from Phe^{406} to Arg^{406} completely inactivated the enzyme, suggesting that Phe^{406} is essential for enzyme activity or for maintaining an important conformation of aromatase (Zhou et al., 1991). However, Kadohama et al. (1993) demonstrated that alteration of Phe^{406} to Arg^{406} significantly changed the cellular and microsomal content of aromatase protein and the enzyme had almost no activity, probably due to structural perturbation of the heme-binding region. Mutation of Ile^{474} to Phe^{474} led to accumulation of more hydroxylation intermediates (19-hydroxyandrostenedione and 19-oxoandrostenedione) than estrone, implying that the binding affinities of the two intermediates for the mutant protein are less than that for wild type (Zhou et al., 1994). Although structural and functional analysis of cytochrome P450 aromatase by means of X-ray crystallographic methods are difficult due to the relative lack

of solubilization of membrane-bound aromatase protein as is the case for most cytochromes P450, a three-dimensional model of human aromatase protein recently was proposed based upon mutagenesis studies and by sequence comparisons with three bacterial P450 enzymes whose protein structures were determined previously (Graham-Lorence et al., 1995).

posttranscriptional modification of aromatase: As shown in Figure 4-3, one or two (only for pig) consensus glycosylation sites were found at the N-terminus of aromatase from all species (except for chicken) whose cDNA sequences have been published. There is evidence that human aromatase is a glycoprotein and that aromatase activity is reduced by 30-40 % after treatment with endoglycosidase (Sethumadhavan et al., 1991). However, disruption of the glycosylation site by changing Thr^{14} to Ala^{14} had no significant change in enzyme activity, although protein expression was lowered as compared with wild type cells (Amarneh et al., 1993). There is evidence suggesting that this activity may be regulated by the phosphorylation of the cytochrome P450 aromatase but not cytochrome P450 reductase component (Bellino and Holben, 1989). Interestingly, a well conserved, putative peptide sequence for phosphorylation by cAMP-dependent protein kinase is found in the peptide determined by exon 9 of aromatase in all species (Figure 4-3) and in all known isoforms of porcine aromatase (Figure 5-3). Since phosphorylation of human cytochrome P450 17α-hydroxylase by a cAMP-dependent protein kinase increases this enzyme's activity (Zhang et al., 1995), the biochemical and physiological significance of the consensus peptide sequence in aromatase should be examined.

other activities of aromatase: It seems likely that multiple functional enzyme activities are commonly found for steroidogenic enzymes. Recently, the estrogen-2-hydroxylase

(catechol estrogen synthetase) activity as well as androgen 19-,1β-, and 2β-hydroxylase activity of cytochrome P450 aromatase was demonstrated in term human placental microsomes and in expressed human term placental aromatase (Osawa et al., 1993). Interestingly, porcine blastocysts secrete large amounts of unknown phenolic compounds whose biological functions have never been investigated (Gadsby et al., 1980; Fischer et al., 1985). In addition, estrogen-2/4-hydroxylase activity (2-hydroxylase activity is nine times higher than 4-hydroxylase activity) is also observed in pig blastocysts, this activity is stimulated by NADPH, and is maximal on Days 12 and 13 (Mondschein et al., 1985). Estrogen 15α-hydroxylase activity with maximal activity on Days 12 and 13 also was detected in porcine blastocysts (Chakraborty et al., 1990). Aminoglutethimide and 4-hydroxyandrostenedione, two inhibitors of aromatase, did not inhibit activity of Estrogen-15α-Hydroxylase activity or Estrogen-2/4-Hydroxylase (Chakraborty et al., 1990). Thus, the three different enzyme activities (15α-hydroxylase > 2-hydroxylase > aromatase) are highly correlated and peak at Days 12 and 13 of pregnancy in pig blastocysts. Interestingly, the two isoforms of aromatase (33F and A10 clones) isolated from pig blastocyst in this study are highly expressed in embryos and are quite different from the placental aromatase isoform. This suggests that the embryonic aromatase isoforms may have additional enzyme activities, in addition to the classical aromatase activity.

Developmental Expression of Cytochrome P450 Aromatase Activity

Blastocysts of a number of mammalian species; rat (Day and Dickmann, 1974), hamster (Dickmann and Sen Gupta, 1974), rabbit (Hoversland et al., 1982; George and

Wilson, 1978), pig (Perry et al., 1973; Gadsby et al., 1980), horse (Zavy et al., 1979), donkey (Heap et al., 1991), camel (Skidmore et al., 1994), and human (Edgar et al., 1993), are known to produce steroids in varying amounts during the periimplantation period. Most of this evidence was provided by biochemical analysis of steroidogenic enzyme activity or by immunolocalization of steroidogenic enzymes or steroids in blastocysts. However, there is still controversy for some species whether periimplantation blastocysts or conceptuses have steroidogenic activity or not, mainly due to the extremely small size of embryos during this period and the difficulty in distinguishing between embryo-originated steroids and steroids diffused from a maternal source. Not much recent progress has been made in unraveling the nature of the temporal and developmental expression of steroidogenic enzymes and their mRNAs in embryos other than those from the pig. Pigs are a good model in which to study the transient expression and physiological consequences of steroidogenic enzyme gene activity in the late periimplantation stage embryo.

aromatase activity in pig blastocysts: Perry et al. (1973) first demonstrated the conversion of estrogens (E1, estrone and E2, estradiol-17β-oestradiol) from two different androgen substrates (androstenedione and dehydroepiandrosterone, respectively) in porcine blastocysts (Days 14, 15, and 16). Since then, steroidogenic activity of pig embryos has been intensively investigated by many researchers. Pig blastocysts show extremely rapid morphological changes (blastocyst elongation) on Days 11 and 12 of pregnancy. Spherical forms of embryos (3-10 mm) are transformed into filamentous forms (> 100 mm) via the intermediate tubular stage (3-10 mm) (Perry and Rowlands, 1962; Anderson, 1978). High aromatase activity was detected in pig embryos on Day 12 of pregnancy and tubular forms

of embryos exhibited the highest aromatase activity as compared to spherical and filamentous forms (Heap et al., 1979; Fischer et al., 1985; Hofig et al., 1991). Consistent with the temporal increase in aromatase activity in pig blastocysts on Day 12 of pregnancy, the estrogen content in elongating blastocysts (E1, 2062 pg and E2, 2051 pg, respectively) was higher than in spherical (E1, 642 pg and E2, 507 pg) and filamentous (E1, 404 pg and E2, 356 pg) forms (Pusateri et al., 1990). Greater expression of aromatase mRNA in Day 12 than Day 15 conceptuses and in concordance with the aromatase protein content was also demonstrated (this study and Ko et al., 1994). The transiently high expression of aromatase mRNA (Green et al., 1995) as well as protein (Conley et al., 1992) which is maximal in elongating blastocysts (tubular form) was also demonstrated. The aromatase protein was immunolocalized primarily to the inner cell layer (endoderm) of the trophoblast (Conley et al., 1994; Ko et al., 1994). Therefore, it is likely that the transiently high production of estrogens by porcine blastocysts, in parallel with high expression of cytochrome P450 aromatase mRNA and protein, is concomitant with the initial stage of a highly developmental-specific event in pig embryology.

The total estrogen content of uterine luminal fluid (ULF) in pregnant sows is greater than for cyclic animals and is increased in ULF containing tubular blastocysts (E_1, 0.8 ng; E_2, 2.3 ng) as compared to ULF containing spherical blastocysts (E_1, 0.5 ng; E_2, 0.6 ng) and continues to increase in the ULF containing filamentous blastocysts (E_1, 2.8; E_2, 4.4 ng) but declines by Day 14 (E_1, 0.6; E_2, 0.4 ng) (Geisert et al., 1982a). Similar patterns of change in estrogen content in ULF were reported by Ford et al. (1982a). The concentration of estradiol-17β differed between uterine arterials and uterine veins in pregnant sows on Days

11 and 13. Concentrations of estradiol-17β, but not estrone, in uterine venous blood on Days 11 and 13 of pregnancy exceeded that for uterine arterial blood (Ford et al., 1982a). This result indicated a higher content of estradiol-17β than estrone in ULF during Days 11 and 13 of pregnancy. This agreed with the findings reported by Geisert et al. (1982a) that the E_2 content exceeded that of the E_1 content in ULF containing tubular or filamentous blastocysts. It should be noted that a several times greater amounts of E_1 than E_2 was produced by pig blastocysts using either androstenedione or testosterone precursors (Perry et al., 1973; Perry et al., 1976; Gadsby et al., 1980). It is likely that pig blastocysts produce estrone (E_1) as the major estrogen and that E1 is converted into estradiol-17β (E_2) by 17β-hydroxysteroid dehydrogenase (17β-HSD), mainly in the uterine endometrium, although pig blastocysts are also known to exhibit 17β-HSD activity. Thus, endometrial 17β-hydroxysteroid dehydrogenase may play a significant role in producing the potent estrogen (E_2) using as precursor of the weaker estrogen (E_1), the former of which may modulate the uterine microenvironment for embryonic growth and development.

It is not obvious which is the direct precursor of embryonic estrogens in the pig. Blood progesterone levels in pregnant sows increased to a peak value of 35.4 ng/ml on day 12 when blastocysts secrete high amounts of estrogens and then declined to 17.2 ng/ml on day 24 of pregnancy (Guthrie et al., 1972). Ovariectomized sows did not maintain pregnancy, whereas the animals that received daily progesterone injections remained pregnant through Day 12 (Heap et al., 1981), indicating that maternal ovary-derived progesterone may be a major precursor for embryonic estrogen synthesis. However, maintenance of pregnancy to Days 20-21 post-coitum was observed in ovariectomized sows treated with MPA

(medroxyprogesterone acetate), a non-aromatizable progesterone, suggesting that maternal progesterone originating from the ovary is not essential for the synthesis of estrogens by pig blastocysts (Heap et al., 1981). It is possible that steroids derived from other tissues, such as maternal adrenal glands and endometrium, may be utilized as precursors for embryonic estrogen synthesis. While expression of 17α-hydroxylase mRNA and protein (Conley et al., 1992; Conley 1994; Green et al., 1995) was demonstrated in pig blastocysts during the periimplantation period, expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) was not detected by western blot analysis (Conley et al., 1992), although 3β-HSD activity was demonstrated in Day 12 pig blastocysts by histochemical analysis (Flood, 1974). It should be noted that pig blastocysts convert dehydroepiandrosterone (DHA) into estrogens (Perry et al., 1973; Perry et al., 1976; Gadsby et al., 1980), suggesting the presence of DHA enzyme activity in the blastocyst.

aromatase activity in other blastocysts: Biosynthesis in Day 6 rabbit blastocysts of cholesterol and pregnenolone from acetate was first reported by Huff and Eik-Nes (1966). Although the validity of that result is not yet clear based upon further investigation by others, that experiment initiated subsequent research concerning the synthesis of steroid hormones by blastocysts of other mammalian species, including the pig. Transient activities of 3β-HSD and 17β-HSD enzymes and content of estradiol-17β were detected in the late blastocyst stage, preimplantation rabbit embryo (144 hr), and these enzyme activities were greater in the embryonic disc than in the trophoblast (Dickmann et al., 1975; Jeorge and Wilson, 1978). On the other hand, Borland et al. (1977) demonstrated that radiolabeled progesterone and estradiol diffused into the blastocoel cavity of the blastocyst suggesting

that rabbit blastocysts did not synthesize the steroids but instead obtained these from maternal uterine fluid. However, transient aromatase activity with the Km value of 0.77 μM and inhibition by aromatase inhibitor was detected in preimplantation rabbit blastocysts consistent with the previous findings of transient enzyme activity for 3β-HSD and 17β-HSD (Hoversland et al., 1982). Thus, it seems likely that the preimplantation rabbit blastocyst produces estrogens by temporal expression of an aromatase gene as is the case for pig blastocysts.

There has been no direct evidence for the presence of aromatase activity in rat blastocysts. However, the presence of 3β-HSD activity in the extra-embryonic ectoderm and inner cell mass of preimplantation rat blastocysts was demonstrated by means of a histochemical method (Dickmann and Dey, 1974; Dey and Dickmann, 1974). In contrast, 3β-HSD activity was not detected in preimplantation rat blastocysts but in postimplantation blastocysts (Sherman and Atienza, 1977). These results indicate that steroidogenic activity is present in rat blastocysts during the periimplantation period. Not only 3β-HSD, but also 17β-HSD activities were detected in preimplantation hamster embryos, implying the possibility of the presence of aromatase activity in hamster blastocysts as well (Dickmann and Sen Gupta, 1974). Human embryos as early as Days 5-8 post-fertilization were found to secrete estradiol-17β and progesterone, and this secretion reached a peak between Days 8 and 11 (Edgar et al., 1993).

High aromatase activity was found in elongating camel blastocysts (Days 10-33) and the major form of estrogen secreted was estradiol-17β, which contrasts with the higher production of estrone by pig blastocysts (Skidmore et al., 1994). Equine blastocysts on Day

8 secrete estradiol-17β during incubation (243 pg/5ml/hour) and this increased until Day 20 (108,763 pg/ 5ml /hour) due to the increase in blastocyst weight. A lesser amount, but similar pattern of secretion, was also detected for estrone (Zavy et al., 1979). Consistent with the above, very high amounts of estrogens (a maximum of 70,000 pg/ml) in blastocoelic fluid of equine blastocysts were detected with a high degree of individual variation noted (Flood et al., 1979). However, it was found that 17α-hydroxyprogesterone but not estradiol was the major steroid synthesized by the equine blastocyst between Days 7 and 14 (Goff et al., 1993). Most recently, it was demonstrated that there is high aromatase mRNA expression in Day 14 equine embryos (see Chapter 4), implying the presence of aromatase activity in these blastocysts. No distinguishable embryonic steroidogenic activity was detected for several other species including the sheep, cow, roe deer, ferret, cat, and plains viscacha (Gadsby et al., 1980).

In summary, the transient steroidogenic activity in pre- or periimplantation blastocysts is a common feature for several mammalian species, although some controversy still remains for some species as described above. It seems likely that aromatase activity is present in rabbit, pig, horse, and camel embryos. The lack of more sensitive methods to detect steroidogenic enzyme activity and gene expression has limited more detailed investigations to clarify and elucidate the nature of developmental expression of steroidogenic activity. However, the recent advances in molecular biological technology such as RT-PCR provide tools to obtain sure answers as demonstrated for porcine cytochrome P450 aromatase in blastocysts and described in this study.

Structure and Regulation of the Cytochrome P450 Aromatase Gene

cDNA cloning: A partial human aromatase cDNA clone was first isolated from an expression library of human placental mRNA using polyclonal antibody raised against aromatase (Evans et al., 1986) and subsequently sequenced (Simpson et al., 1987). Chen et al. (1988) also obtained a partial cDNA clone from a human placental cDNA library using a unique degenerate oligonucleotide probe designed from the N-terminal sequence of human aromatase (Chen et al., 1986). Both of these cDNA clones contained protein-encoding and 3' untranslated sequences but were missing a portion of the sequence encoding the N-terminus of the protein. Soon thereafter, full-length cDNA clones encoding the 503 amino acids (A.As) of human aromatase were isolated from human placental cDNA libraries by four different groups (Corbin et al., 1988; Harada, 1988; Pompon et al., 1989; Toda et al., 1989). A full-length cDNA clone encoding 507 amino acids of chicken ovary aromatase was isolated using the human aromatase cDNA probe (McPhaul et al., 1988). Later, human and chicken aromatase cDNA probes were used to isolate cytochrome P450 aromatase cDNA clones from a variety of species including the rat (508 A.As, Hickey et al., 1990), mouse (503 A.As, Terashima et al., 1991), rainbow trout (522 A.As, Tanaka et al., 1992), Zebra finch (509 A.As, Shen et al., 1994), catfish (509 A.As, Trant,1994), and cow (503 A.As, Hinshelwood et al., 1993; Vanselow and Furbaβ, 1995). A variant aromatase transcript also was isolated from bovine placenta (Furbaβ and Vanselow, 1995). This clone contains the homologous sequence (89-98%) corresponding to exons II, III, V, VIII and IX of bovine aromatase. However, the Exon IV segment is replaced by a bovine repeated sequence and

exons VI and VII were deleted. The finding of numerous translational stop codons in all reading frames suggests that this transcript does not encode a functional protein. Recently, two different aromatase cDNA clones encoding isoforms of porcine aromatase were isolated from ovary (501 A.As, Corbin et al., 1995) and term placenta (503 A.As, Corbin et al., 1995). Therefore, the pig is the only species to date in which multiple isoforms of aromatase have been identified. It is important to note that all aromatase cDNA clones have been isolated from a single source of tissue for each species, typically either ovary or placenta (with the exception of the pig). Exhaustive cloning from multiple tissues has not been employed prior to the conduct of studies described in this dissertation.

genomic DNA structure: The genomic DNA clones encoding cytochrome P450 aromatase were isolated from human genomic DNA libraries by three different groups (Means et al., 1989; Harada et al., 1990; Toda et al., 1990). This gene spans at least 75 kb in length, consists of more than 10 exons, with the translational initiation and termination codons located in exons 2 and 10, respectively. Surprisingly, the intron 1 between the placenta-specific first exon (EI-1) responsible for the 5' untranslated sequence in placental aromatase transcripts and exon 2 (EII) is at least 35 kb in size, and linkage between these two exons by overlapping genomic clones has been unsuccessful. Thus, the gene encoding human cytochrome P450 aromatase is larger than other members of the cytochrome P450 superfamily. The presence of more than one chromosomal gene for human aromatase was initially suggested from the results of Southern blot analysis (Chen et al., 1988). However, evidence for the existence of only a single human gene was provided from results of mapping of genomic aromatase clones and Southern blot analysis conducted by others (Means et al.,

1989; Toda et al., 1990). The cytochrome P450 aromatase gene was localized on human chromosome 15 (Chen et al., 1988).

Previously, the chicken (Matsumine et al., 1991) and medaka fish (Tanaka et al., 1995) were the only species other than the human in which the structure of the aromatase gene was characterized. The chicken gene is composed of nine coding exons and is ~ 30 kb in size. The results from genomic Southern blot analysis indicated the presence of only a single aromatase gene in this species. There is evidence for the existence of a single aromatase gene in the mouse (Terashima et al., 1991). The size of the medaka P450 aromatase gene is only 2.6 kb in length and consists of nine protein-coding exons (Tanaka et al., 1995). The bovine genome contains a pseudogene which is responsible for the variant aromatase transcript described above (Furbab and Vanselow, 1995). The number of protein-encoding exons and the splice junctions are highly conserved for the human, chicken, and medaka fish (Tanaka et al., 1995).

alternative splicing of 5' untranslated exons: The presence of at least two untranslated exons (EI-1 and EI-2) upstream of the human placental aromatase gene, which are alternatively spliced, was first suggested from the results of RT-PCR, Northern blot analyses and S1 nuclease protection assays. An additional promoter upstream of another untranslated exon was suggested to be responsible for transcriptional regulation of aromatase transcripts in adipose stromal cells (Mahendroo et al., 1991). Subsequently, it was found that the expression of aromatase mRNA in human corpus luteum is regulated by an additional promoter (PII), which is located just 5' of coding exon II of the human aromatase gene. From these results, it was suggested that: a) the tissue-specific regulation of the human aromatase

gene is in part the consequences of the utilization of tissue-specific promoters, and b) the ovarian promoter (PII) is the ancestral promoter for this gene because avian species do not have a placenta (Means et al., 1991). An additional aromatase cDNA was obtained from dexamethasone-treated human fibroblasts by means of 5' RACE (rapid amplification of cDNA ends) and this clone contained a unique exon 1 (Harada, 1992). A new type of full-length cDNA clone encoding human aromatase with 109 bp of unique 5' untranslated exon sequence was isolated from a human placental cDNA library and the exon corresponding to this 109 bp sequence was localized at ~ 10 kb downstream of exon I-1 (Toda and Shizuta, 1993). Several additional 5' untranslated exons were subsequently identified from different tissues and all of these were localized between exon I.1 and exon II in the human chromosomal gene (Harada et al., 1993; Mahendroo et al., 1993; Toda et al., 1994). Interestingly, differential expression of two distinct 5' exons in adipose tissue was found; one exon is prevalent in breast cancer patients and the other in normal tissue, and a developmental change of multiple exons 1 for liver aromatase transcripts was demonstrated (Harada et al., 1993). Mahendroo et al. (1993) found that glucocorticoids stimulate the production of transcripts containing exon I.4, suggesting that the hormonal environment may, in part, play a role in the selection of specific 5' exons included in the mature aromatase transcripts. The most recently described new exon (1f) is specific to the brain and its upstream site localization indicated that the human aromatase gene has a length of more than 80 kb (Honda et al., 1994). Therefore, the gene for human cytochrome P450 aromatase contains at least seven 5' untranslated exons in addition to the nine coding exons.

The 5' untranslated sequence of bovine placental aromatase cDNA (Vanselow and Furbab, 1995) is unique and different from the 5' upstream sequence of bovine exon II (Hinshelwood et al., 1995) and the 5' sequences of other species, suggesting the occurrence of alternative splicing of 5' exons of the aromatase gene in this species. The demonstration of the differences in 5' untranslated mRNA sequences of placental and ovary aromatase genes in the cow and horse (Hinshelwood et al., 1995) suggests that alternative usage of multiple 5' untranslated exons is common at least for mammalian species. As in the human, a brain-specific exon 1f was found in the monkey brain (Yamada-Mouri et al., 1995). There is no information regarding the molecular mechanisms of alternative splicing of 5' untranslated exons in this gene. An understanding of these mechanisms is important to elucidating the nature of the tissue-specific regulation of this gene in the future.

regulatory mechanisms of gene expression: cAMP was stimulatory to steady-state levels of aromatase mRNA and estradiol biosynthesis in rat granulosa cells of preovulatory follicles, but the expression of aromatase was maintained by a cAMP-independent mechanism(s) after LH/hCG-induced luteinization (Hickey et al., 1990). Transient transfection assays indicated that promoter region sequences, located between -176 and -31, of the rat aromatase gene were required for cAMP-induction in primary cultures of granulosa cells and for expression in rat R2C Leydig cells. Binding of nuclear extracts from granulosa cells and R2C Leydig cells to a radiolabeled -176/13bp fragment with competition by cold competitor fragment as well as by a shorter region (-90/-66 bp) containing putative SF-1 (steroidogenic factor-1) binding site (AGGTCA) was demonstrated by gel mobility shift assay (Fitzpatrick and Richards, 1993). SF-1 sites are found in the promoter regions of most

of the cytochrome P450 steroidogenic enzyme genes analyzed to date (Moroshi et al., 1992; Omura et al., 1995; Parker and Schimmer, 1995). The failure of purified CREB to bind this fragment suggests that the protein(s) binding to the -90/-66 bp fragment and mediating cAMP regulation of the aromatase gene is not a CREB nuclear protein. Specific binding of expressed SF-1 to this region was demonstrated by gel retardation assay (Lynch et al., 1993). An additional region (-161/-138 bp) that is required for cAMP regulation was also identified. Specific binding of nuclear extracts from granulosa and R2C cells to the -161/-138 bp region was also demonstrated. It was further confirmed by binding of expressed CREB to the CRE-like sequence and retarded migration of granulosa cell protein-aromatase DNA binding complex after treatment of CREB antibody (Fitpatrick and Richards, 1994). The activity of a CAT construct containing -534/+105 of rat aromatase promoter was abolished by the A-kinase specific inhibitor, suggesting that activation of protein kinase A is obligatory for transcriptional activation of this promoter region (Orly et al., 1996). Moreover, cAMP-stimulated transcription was lost upon deletion of the -278/-100 bp region containing the putative SF-1 site in human ovary promoter (PII). SF-1 mRNA, protein levels, and DNA binding activity are increased in forskolin-treated luteal cells. Binding of SF-1 to -278/-100 was demonstrated by supershift electrophoretic mobility shift assay and UV cross-linking. Therefore, these data suggest a mechanism of cAMP-induction of transcription of the SF-1 gene (Michael et al., 1995). It was suggested that a CRE-like sequence present in the human PII region at -211 bp may act together with the hexameric element for the cAMP-dependent stimulation of transcription as is the case for the rat promoter (Michael et al., 1995). The ovarian aromatase gene promoter regions of several other species including the bovine

(Hinshelwood et al., 1995), and medaka (Tanaka et al., 1995) gene also contain potential SF-1/Ad4BP sites, suggesting that the basic structural organization of this genes and the regulatory mechanisms for PII are well conserved through-out vertebrates.

Northern blot analysis demonstrated that expression of the aromatase gene in human choriocarcinoma (BeWo) is remarkably stimulated by treatment of cells with 12-O-tetradecanoyl-phorbol 13-acetate (Toda et al., 1990). The -500/-243 region in the major placental promoter contains a negative cis-acting element, whereas the -242/-183 region is required for efficient transcriptional activity in response to phorbol ester (Toda et al., 1990). Subsequent experiments indicated that the region between -242 and -166 contains a cis-acting enhancer element, which increased promoter activity in a manner dependent on copy number but independent of position and orientation. The nuclear extract prepared from BeWo cells specifically interacted with the cis-acting enhancer element, although neither DNase I foot-printing nor methylation-interference analysis showed any specific nucleotide sequence responsible for binding (Toda et al., 1992). Two additional cis-acting elements, designated as hATRE-1 (human aromatase cytochrome P450 gene transcriptional regulatory element-1) and hATRE-2 were located between -2238 and -2214, and between -2141 and -2098, respectively, upstream of the major placental aromatase gene promoter. Transient expression analysis in choriocarcinoma cells showed that hATRE-1 repressed the expression of a reporter gene, whereas hATRE-2 enhanced this in response to 12-O-tetradecanoylphorbol 13-acetate. Eletrophoretic mobility shift analysis indicated that nuclear binding factors specific to hATRE-1 and hATRE-2 were present in BeWo cells (Toda and Shizuta, 1994). Recently, it was demonstrated that nuclear factor interleukin-6 (NF-IL6),

which was originally identified as a transcription factor responsible for induction of the human IL-6 gene, was the major nuclear factor binding to hATRE-2 in Be Wo cells. Transient transfection analysis indicated that binding of NF-IL6 to hATRE-2 was required in BeWo cells for transcriptional activation by this cis-acting element and disruption of the NF-IL6 binding site resulted in the disappearance of the transcriptional enhancing activity of the element (Toda et al., 1995). However, exactly how constitutively expressed NF-IL6 in BeWo cells selectively stimulates aromatase gene expression remains to be determined. The induction of aromatase gene expression via treatment with different types of phorbol acetate was also demonstrated using JAR cells, another human placental choriocarcinoma cell line (Wang and Chen, 1994). A recent study demonstrated that DNA-sequences between -301 and +22 bp of the placental-specific exon-I are sufficient for placental expression of aromatase. In gel mobility shift assays, three separate domains (C2, C3, and C4 domains) in this region form specific binding complexes with nuclear extracts from JEG-3 choriocarcinoma cells. Two of the binding domains (C2 and C4) that form major complexes in gel shift assay compete with each other and with a DNA fragment containing the trophoblast-specific element (TSE), which is derived from the enhancer region of the human chorionic gonadotropin α-subunit gene. These results further indicated the two binding domains were recognized by the same trans-acting factor (Yamada et al., 1995). The importance of the region between -242 and -183, which contains the C3 domain, was previously shown by Toda et al. (1990).

Promoter sequence specific to exon 1.4 does not contain a TATA-like sequence and is known be utilized in transcripts expressed in adipose stromal cells tissue and fetal liver

(Zhao et al., 1995a). The -550/+10 bp promoter region construct expressed CAT activity after a putative silencer element was deleted and this expression was induced about 3-fold by dexamethasone. Transfection of the -330/+170 bp construct, which contained an upstream glucocorticoid response element (GRE) as well as an Sp1-like sequence in the untranslated exon 1.4, resulted in an 8-fold stimulation of expression of CAT activity by dexamethasone. Mutation of either the GRE or Sp1 binding sites or both in the -330/+170 bp construct resulted in the loss of dexamethasone-induced CAT reporter gene expression (Zhao et al., 1995a). Gel retardation analyses using nuclear extracts of human adipose stromal cells cultured in the presence of serum and dexamathasone, and an oligonucleotide probe containing the GRE and Sp-1 binding sequence showed that specific proteins were present in the nuclear extracts that bound to the GRE and the Sp1-like sequences (Zhao et al., 1995a). Subsequent experiments demonstrated that the stimulatory effects of serum (in the presence of dexamethasone) could be replaced by interleukin(IL)-11, leukemia inhibitory factor, and oncostatin-M, as well as by IL-6. Stimulation of the cells by these cytokines led to rapid phosphorylation of Jak1 kinase and STAT3 transcription factors. Phosphorylated STAT3 was bound to the GAS (interferon-γ activation site) sequence in the I.4 promoter region, and mutagenesis or deletion of the GAS sequence led to complete loss of reporter gene expression (Zhao et al., 1995b). It should be noted that activation of this pathway of expression by these cytokines is absolutely dependent on the presence of glucocorticoids. A variety of other growth factors including EGF (epidermal growth factor), PDGF (platelet-derived growth factor), FGF(fibroblast growth factor), growth hormone, prolactin, and IGF-I had no effect on aromatase gene expression in adipose stromal cells (Zhao et al., 1995b).

Therefore, it is likely that a) cAMP induced by FSH is the major regulatory factor interacting with CRE sequence in the ovary promoter and modulates the expression or action of SF-1, b) glucocorticoids are the major regulatory hormone controlling the expression of the aromatase gene in adipose stromal cells in combination with factors such as IL-6, and c) placental aromatase expression is under the control of a protein kinase C pathway although its upstream regulatory factor(s) has not been identified.

gene regulation by growth factors and cytokines: The rate of synthesis of cytochrome P450 aromatase in human adipose stromal cells was stimulated by cAMP. This stimulation was attenuated by epidermal growth factor and was potentiated by phorbol esters with comparable changes in the levels of mRNA as well as activity of aromatase (Evans et al., 1987). Epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) markedly inhibited the cAMP-stimulated aromatase activity in adipose stromal cells. However, insulin-like growth factor I (IGF-I) in the presence of dexamethasone and serum stimulated aromatase activity over that seen with dexamethasone alone (Simpson et al., 1989). Treatment with IGF-I increased the FSH-stimulated accumulation of estrogen and FSH-induced aromatase activity in cultured rat granulosa cells (Adashi et al., 1985). IGF-I increased aromatase activity with an increase of mRNA expression in human ovarian granulosa cells, while EGF had no effect on these parameters when added alone, but markedly inhibited the action of FSH to stimulate aromatase mRNA expression (Steinkampf et al., 1988). Incubation with IGF-I alone caused an increase in E_2 production comparable to that caused by FSH and coincubation with FSH and IGF-I augmented E_2 levels more than either hormone alone, suggesting that these factors were synergistic in human granulosa and

granulosa luteal cells (Erickson et al., 1989). Similar results were also observed in polycystic human ovaries (Erickson et al., 1990). Aromatase activity of day 12 but not day 10 pig conceptuses was increased by IGF-I with no change observed by addition of insulin (Hofig et al., 1991). It was shown that addition of IGF-I increased the aromatase mRNA level in filamentous forms of pig conceptuses but decreased aromatase mRNA levels in spherical forms of conceptuses without altering mRNA levels for IGF-1 receptors in both type of conceptuses (Green et al., 1995). In contrast to the stimulatory effects of IGF-I described above, incubation of human placental cytotrophoblasts with insulin or IGF-I significantly inhibited the conversion of androstenedione to estrogens. Treatment with a monoclonal anti-IGF-I antibody prevented the suppression of aromatase activity by IGF-I, but stimulated P450 SCC activity (Nestler, 1987). On the other hand, IGF-I stimulated the 3β-hydroxysteroid dehydrogenase activity of human placental cytotrophoblasts (Nestler, 1989). Similarly selective inhibitory effects on aromatase were also observed for IGF-II (Nestler, 1990). EGF, but not FGF, inhibited the gonadotropin stimulation of estrogen and testosterone production by primary cultures of ovarian granulosa and testicular Leydig cells. EGF also inhibited gonadal steroidogenesis induced by cholera toxin and (Bu)2cAMP (Hsueh et al., 1981). TGFβ decreased the aromatase activity with a concomitant decrease in aromatase protein and cAMP-induced mRNA levels of aromatase in human fetal hepatocytes (Rainey et al., 1992). However, TGF-β enhanced FSH-induced aromatase activity in rat granulosa cells (Dorrington et al., 1993).

Interleukin-1(IL-1) inhibited the FSH induction of aromatase activity with reduced cAMP accumulation in porcine granulosa cells (Yasuda et al., 1990). In rat Sertoli cells, IL-

1β, but not IL-1α, inhibited FSH-stimulated aromatase activity with a significant inhibition of cAMP production and the inhibitory activity of IL-1β was blocked by a specific IL-1β antiserum (Khan and Nieschlag, 1991). On the other hand, human cytotrophoblasts treated with human IL-1α or IL-1β consistently increased aromatization of androgens to estrogens, suggesting that endogenously produced IL-1(β) may function to enhance cytotrophoblast aromatase activity (Nestler, 1993). The demonstration that binding of nuclear factor interleukin-6 (NF-IL6), which was originally identified as a transcription factor responsible for induction of the human IL-6 gene, to hATRE-2 is required for TPA-induced enhancement of CYP19 gene expression is intriguing (Toda et al., 1995). Therefore, the effect of growth factors is likely to depend on the tissue context and may indirectly involve the expression or activity of other tissue-specific factors.

Definition of the Problem and Implications

There has been a significant amount of progress made in elucidating cytochrome P450 aromatase structure, function and genetic regulation since aromatase activity was first identified in human placenta in the 1950's. This enzyme has received much attention not only because of its importance in reproductive processes but also because of its involvement in human cancers of the breast and endometrium. Human placenta has been the most popular tissue used for biochemical studies of this enzyme, probably due to its easy accessibility. There was some controversy regarding the presence of multiple isoforms of aromatase enzyme based upon previous biochemical studies. The demonstration of multiple activities for a single isoform of human placental aromatase resolved this controversy temporarily.

However, the present results still cannot rule out the possibility of the presence of aromatase isozymes that possess different activities depending upon the steroid precursors used. The recent identification of multiple isozymes for other steroidogenic enzymes and of human placental aromatase as a catechol estrogen synthetase have revitalized the question of the existence of multiple aromatase isoforms. It should be noted that either the placenta or the ovary are the only tissues utilized for the cloning of aromatase cDNA for all species so far. The presence of only a single aromatase gene in a several species was suggested by genomic Southern blot analysis.

It is still controversial for some species whether aromatase is actually expressed in preimplantation embryos. The major problem has been the lack of a sensitive method to detect aromatase in blastocysts. With the advent of molecular biological techniques, tools to detect expression of the aromatase gene in blastocysts are now available. The identification of unknown phenolic compounds and the temporally high activity of catechol synthetase in parallel with aromatase in porcine blastocysts support the concept that pig embryo aromatase may have additional enzyme activities. The molecular cloning of aromatase cDNA from mammalian blastocysts will provide answers regarding these issues. The accumulated body of physiological, biochemical, and molecular biological data regarding the expression of the aromatase enzyme in pig blastocysts will provide a foundation for unraveling the molecular mechanisms underlying expression and functional role of the corresponding gene.

CHAPTER 3
EXPRESSION OF THE CYTOCHROME P450 AROMATASE GENE IN ELONGATING PORCINE BLASTOCYSTS

Introduction

Studies concerning the regulation of estrogen synthesis and secretion by mammalian preimplantation embryos such as those from the pig (Perry et al., 1973), horse (Zavy et al., 1979) and human (Edgar et al., 1993) have lagged behind comparable studies for the ovary and the placenta, in part due to the latter tissues' higher capacity for steroid hormone production (Miller, 1988). The amounts of estrogens synthesized by pre-implantation embryos during the critical period of pregnancy establishment, however, are significant and are altered in distinct fashion as embryonic development proceeds (Perry et al., 1973; Pusateri et al., 1990; Edgar et al., 1993). By forming specific complexes with their nuclear receptors (Wahli and Martinez, 1991; Baniahmad and Tsai, 1993), embryo-derived estrogens may elicit transcriptional activation of maternal and/or embryonic genes such as those encoding growth factors, ensuring the timely participation of their respective gene products in the normal progression of embryo development. This hypothesis is consistent with the demonstrated expression of a wide array of estrogen-induced growth factors in the uterine environment of pre- and early postimplantation embryos (Pollard, 1990; Simmen and Simmen, 1991; Simmen et al., 1995).

Our laboratories have utilized the preimplantation pig conceptus (embryo and associated membranes) as a model system to elucidate the interrelationships of embryo-derived estrogens and the insulin-like growth factors (IGFs) in the control of embryonic development *in utero*. Previous studies have documented the temporal correlation of uterine-derived IGF-I during early pregnancy coincident with conceptus estrogen production and elongation of developing conceptuses (Simmen et al., 1992). In line with the possibility that these embryo-derived estrogens may trigger increased local concentrations of growth factors in the maternal-embryonic environment, we have demonstrated that these estrogens induce the endometrial secretion, but not synthesis of IGF-I (Simmen et al., 1990), which increased the levels and/or activity and mRNA of the steroidogenic enzyme, aromatase cytochrome P450 in conceptuses (Hofig et al, 1991; Green et al., 1995). It is not known however, if IGF-II, the other closely related member of the IGF family, is also involved in this developmental event.

Estrogens constitute the biological signal for maternal recognition of pregnancy in the pig (Flint et al., 1979). Although the nature of this signal differs among mammalian species, with chorionic gonadotrophin for human (Fishel et al., 1984) and trophoblast interferon for cow and sheep (Roberts et al., 1992) embryos, it is clear that the timely and appropriate production of these signals is critical for embryo development. Estrogens produced by the late pre-implantation stage porcine conceptuses are paracrine effectors of endometrial function (Simmen et al., 1990; Simmen et al., 1995). The reported coincident secretion by human embryos of estrogens and chorionic gonadotrophin (Edgar et al., 1993) is consistent with a potential correlation between estrogens and other pregnancy-associated biological

signals for the maintenance of a maternal environment receptive to implantation and subsequent development of the embryo.

The present study investigated the temporal regulation of pig conceptus aromatase cytochrome P450 expression at the levels of mRNA and protein, and compared these with amounts of IGFs in the uterine microenvironment. The results add further support to the hypothesis that temporal induction of aromatase P450 gene expression is one mechanism by which the actions of uterine IGFs can regulate coordinate embryonic and endometrial function during development.

Materials and Methods

Animals

Pigs were bred at estrus (Day 0) after two estrous cycles of normal duration (17-22 days) were observed. Animals at Days 10, 11, 12, 15 and 18 of pregnancy were slaughtered at the Meats Processing Facility (University of Florida), and reproductive tracts were immediately removed and placed in ice. Conceptuses were recovered into petri dishes by flushing each uterine horn with 20 ml of sterile 0.9% (w/v) NaCl. The conceptuses and uterine flushings were transferred to sterile 50 ml tubes and separated by low speed centrifugation at 4° C for 10 min. The flushings and tissues were snap-frozen in liquid nitrogen and stored at -80° C until later use.

Cloning of Porcine Aromatase Genomic DNA

Approximately 300,000 bacteriophages of a porcine genomic library made from adult pig liver (Clontech, Palo Alto, CA) were screened by hybridization with a human aromatase cDNA fragment spanning exons 3-10 of the human gene (Chen et al., 1988). The conditions

for the library screening followed previously described protocols (Simmen et al., 1988a). Two positive clones obtained from the primary screening remained positive after additional rounds of rescreening. DNA was prepared from one clone following standard methods (Maniatis et al., 1982) and was further analyzed by restriction endonuclease mapping and Southern blot analysis. A *Hind* III x *Pst* I genomic fragment of approximately 1 kb in length which hybridized to the human cDNA probe in Southern blots was subcloned into the pGEM4Z plasmid vector (Promega, Madison, WI). The resultant plasmid was designated pAROB9-1. The sequence of the DNA fragment was determined in its entirety by the dideoxychain termination method (Sanger et al., 1977) using the Sequenase DNA sequencing kit (United States Biochemicals, Cleveland, OH). Sequence alignment and other analyses was performed using the programs of the Genetics Computer Group (University of Wisconsin). The sequence reported in this chapter has been deposited in GenBank and has accession number L15471.

RNA Preparation and Analysis

RNA was extracted by the guanidinium-thiocyanate method of Puissant and Houdebine (1990). For Northern blot analysis, 30 ug of total cellular RNA was electrophoresed in a 1% agarose/2.2 M formaldehyde gel and transferred to a Biotrans nylon membrane. Filters were prehybridized and hybridized at 50°C as previously described (Simmen et al., 1988a), except that prehybridization and hybridization buffers contained 1 mg/ml yeast RNA. Dot blot analysis of total cellular RNA (10 ug) followed previously described protocols from our laboratories (Simmen et al., 1992a), using Rapid hybridization buffer (Amersham, IL) at an incubation temperature of 70° C for overnight. The single-

stranded, antisense RNA probe used for hybridization was generated by *in vitro* transcription of *Sal* I-cleaved pAROB9-1 using T7 RNA polymerase in the presence of ^{32}P-CTP (Amersham, Arlington Heights, IL), using the Maxiscript kit (Ambion, Austin, TX). Filters were sequentially washed in 2 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS at 65°C for Northern blots and 68°C for RNA dot blots, and exposed to X-ray films at -80°C for 2-3 days. Filters were also subjected to phosphorimage analysis to quantify hybridization signals.

Synthetic Peptide and Immunization

The peptide DDVIDGYPVKKGTNI corresponding to a highly conserved region of human, mouse, rat, chicken, rainbow trout (Chen et al., 1988; Corbin et al, 1988; McPhaul et al., 1988; Harada et al., 1990; Hickey et al., 1990; Terashima et al., 1991; Tanaka et al., 1992) and pig (this chapter) aromatase P450 protein and which did not exhibit any significant homologies to other P450 proteins, was synthesized by the ICBR Protein Synthesis Core using an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, CA). The purity of the resultant peptide was confirmed by HPLC and its amino acid composition was verified prior to use. The peptide was coupled to keyhole lymphet hemocyanin (KLH; Sigma, St. Louis, Mo) via a cysteine residue added to the amino-terminal portion of its sequence during synthesis, and 4 mg of the conjugated peptide in complete Freund's adjuvant was injected intramuscularly in two sheep. Subsequent injections of conjugated peptide in incomplete Freund's adjuvant were given every 10 days. Initial determination of antiserum reactivity with the synthetic peptide was determined 10 days after the fourth injection (see below). The antiserum collected after the eighth boost was used for the studies described here.

Aromatase Radioimmunoassay and Sample Preparation

The titer of the aromatase oligopeptide antiserum was determined as previously described for antiserum generated to antileukoproteinase oligopeptide (Simmen et al., 1992b). Following this protocol, dilution of aromatase antiserum to 1:8000 yielded 50% percent binding of added labeled peptide. Aromatase peptide (Arom 16P) was radiolabeled to a specific activity of 62 uCi/ug using $Na^{125}I$ and the chloramine T method (Lee and Hendricks, 1990). Iodinated peptide was purified by LH-20 gel-filtration chromatography.

Tissue extracts were prepared by sonication of conceptus tissues in RIPA lysis buffer [100 mM sodium phosphate, pH 7.2; 10 mM EDTA; 10 mM EGTA; 10 mM NaF; 1% (v/v) Triton X-100; 0.1% (w/v) SDS; 1% (v/v) sodium deoxycholate; 1 mM PMSF; and 200 Kallikrein U aprotinin/ml] at a ratio of 0.5 g tissue/ml buffer. The homogenate was incubated at 4°C overnight with shaking and then centrifuged briefly to remove insoluble material. The supernatant was assayed at volumes of 30-100 ul. Antigen-antibody complexes were precipitated by addition of rabbit anti-sheep immunoglobulin (IgG; Sigma Biochemicals, St. Louis, MO.) at a final dilution of 1:800 and incubation for 1 hr at 4° C. To generate the standard curve for the assay, unlabeled peptide was used at doses ranging from 0.02 to 51.2 ng. The sensitivity of the assay was 0.1 ng/tube and the ED_{50} was 4 ng/tube. The intra- and interassay coefficients of variation were 4.9% and 12.4%, respectively. RIA results were expressed as ng immunoreactive protein per ug DNA. DNA content in tissue extracts was determined by fluorometric assay (Labarca and Paigen, 1980).

Radioimmunoassays for IGF-I and IGF-II

Uterine luminal fluids were acidified with 1% aqueous trifluoroacetic acid and chromatographed in Sep-Pak plus C_{18} columns to remove IGF binding proteins prior to RIA. These protocols have been described in detail previously (Ko et al., 1991; Lee et al., 1991). Approximately 70% and 77% of IGF-I and IGF-II, respectively were recovered by the extraction procedure. Acetonitrile eluates obtained by Sep-Pak chromatography were analyzed for IGF-I and IGF-II immunoreactivity as previously described (Ko et al., 1991; Lee et al., 1991) using polyclonal antiserum against IGF-I or monoclonal antibody against rat IGF-II and recombinant human IGF-I and IGF-II as iodinated tracers and standards. All ULF samples were assayed at the same time to avoid inter-assay variation. Intra-assay coefficients of variation were 3.7% in the IGF-I RIA and 5.5% in the IGF-II RIA.

Statistical Analysis

Conceptus aromatase P450 protein and mRNA levels and ULF IGF concentrations were subjected to one-way analysis of variance (Barr et al., 1979). Orthogonal contrasts were used to test for differences between specific days of pregnancy. Correlation coefficients were derived by the ProcCorr procedures of PC-SAS.

Results

Porcine Aromatase Genomic DNA

To enable the generation of a homologous aromatase P450 cRNA probe for analysis of conceptus tissue mRNA abundance and to obtain a homologous peptide sequence for generation of antiserum, a human aromatase cDNA fragment was used to screen a porcine

genomic DNA library. Two bacteriophages that hybridized to the human cDNA probe were isolated, and the cloned genomic DNA insert from one of the isolated plaques was characterized further. Bacteriophage DNA was subjected to restriction endonuclease mapping followed by Southern blot analysis. The smallest genomic subfragment (*Hind* III x *Pst* I) which hybridized to the human cDNA probe was subcloned in pGEM4Z and sequenced in its entirety. The DNA fragment, designated pAROB9-1, is ~ 1.0 kb in length (Figure 3-1) and contains a 242 bp sequence which exhibits 90% identity with, and exactly the same length as, exon 9 of the human aromatase P450 gene (Harada et al., 1990). The nucleotide sequence of the porcine exon and immediate-flanking intron regions and the deduced primary sequence of the encoded protein are presented in Figure 3-2. Comparison of the deduced amino acid sequence of this region of porcine aromatase with those of other species (Figure 3-3) demonstrated the highest identity (94%) with the analogous region of the human protein. However, significant identity was also observed with the corresponding regions of the rodent (86%), avian (83%) and fish (66%) aromatase proteins. The above described sequence contains the ozols peptide region (Corbin et al., 1988) which is highly conserved among the aromatase P450 proteins of different species.

Developmental Expression of Aromatase mRNA

A high specific activity, single-stranded antisense RNA was generated by *in vitro* transcription of the pAROB9-1 plasmid template in the presence of ^{32}P-CTP and was utilized as a probe in Northern blot analysis to determine the size of aromatase P450 mRNA in elongating conceptuses. A major hybridizing band of 2.7 kb in length was detected in total cellular RNA (30 ug) extracted from Days 12 and 15 conceptuses (Figure 3-4A).

Phosphorimage analysis of this Northern blot indicated 4-5-fold greater levels of aromatase mRNA in Day 12 than in Day 15 conceptus tissues. To examine further the changes in the levels of this mRNA as a function of stage of conceptus development, equal amounts of total RNA prepared from pooled conceptuses of individual pigs at Days 12 (n = 4), 15 (n = 3) and 18 (n = 4) of pregnancy, where n represents the number of pigs, were analyzed by RNA dot-blot hybridization with the porcine aromatase antisense probe. The limitations in size of conceptuses prior to Day 12, which are spherical in form, precluded the analysis of P450 aromatase mRNA at these stages. Day 12 conceptuses were a mixture of tubular and filamentous forms, whereas Days 15 and 18 conceptuses were filamentous. Phosphorimage analysis of the resultant blot (Figure 3-4B) demonstrated that the relative level of aromatase mRNA in Day 12 conceptuses was higher ($P = 0.07$) than for Days 15 and 18 conceptuses, which did not significantly differ from each other. Equivalent amounts of yeast RNA applied to the filter were negative for hybridization signal.

Characterization of Aromatase Oligopeptide Antiserum

Immunization of sheep with a synthetic peptide corresponding to the highly conserved region of porcine, human, rat, mouse and chicken P450 aromatase protein (Figure 3-3) yielded a high titer antiserum that specifically bound the aromatase peptide (Figure 3-5). Unrelated proteins or peptides did not compete for binding to the antiserum even when added at high concentrations. In contrast, proteins from solubilized microsomal membranes prepared from late-pregnant (Day 112) pig placenta and Day 18 pig conceptus tissues exhibited displacement curves parallel to those of the oligopeptide. Western blot analysis conducted by Y. Ko in our laboratory showed that this antibody recognized a protein of Mr

49,000 in tissue extracts prepared from porcine conceptus, placenta, endometrium, and ovary. These results indicated the utility of the generated antibody as a specific reagent for quantifying tissue levels of aromatase protein.

P450 Aromatase Content in Conceptus Tissues

A radioimmunoassay was developed to quantify levels of P450 aromatase protein in conceptuses. For these studies, conceptuses were pooled within each pig at each day of pregnancy and three pigs per day were used as sources of conceptuses. Results which are expressed as pg aromatase protein per ug cellular DNA, are presented in Table 3-1. On a per cell basis, the levels of aromatase protein decreased as a function of stage of development. Highest levels of the aromatase protein were observed in Day 10 conceptuses and were significantly diminished in Days 15 and 18 conceptuses. The values for total DNA (ug) per pig embryo at different days of development have been determined previously (Geisert et al., 1982b; Pusateri et al., 1990). Based on the reported total DNA per embryo of 1 ug (Day 10), 6 ug (Day 11), 10 ug (Day 12), 47 ug (Day 15) and 130 ug (Day 18), respectively, the total content of aromatase P450 in conceptuses was calculated and is shown in Table 3-1. Total conceptus aromatase content increased from Day 10 to Days 11 and 12 and significantly decreased by Days 15 and 18. This temporal pattern followed more closely that of the levels of conceptus estrogens, which peaked at Days 11 and 12, decreased by Day 15 and slightly increased by Day 18 (Stoner et al., 1981; Pusateri et al., 1990).

IGF Concentrations in Uterine Luminal Fluids

The concentrations of IGF-I and -II in ULF as determined by RIA are presented in Figure 3- 6. As previously reported (Letcher et al., 1989), IGF-I levels in ULF were highest

at Day 12 and were significantly ($P = 0.001$) diminished by Day 15 of pregnancy. The levels of ULF IGF-II were low but detectable at Days 10 and 11, were slightly increased by Day 12 and then remained at that level up to Day 18 of pregnancy. No significant correlation was observed between levels of IGF-I nor IGF-II alone with conceptus aromatase production per embryo. However, the decreasing levels of aromatase protein as a function of stage of development were positively correlated ($r = 0.95$; $P = 0.015$) with the decreasing ratio of IGF-I to IGF-II in the ULF.

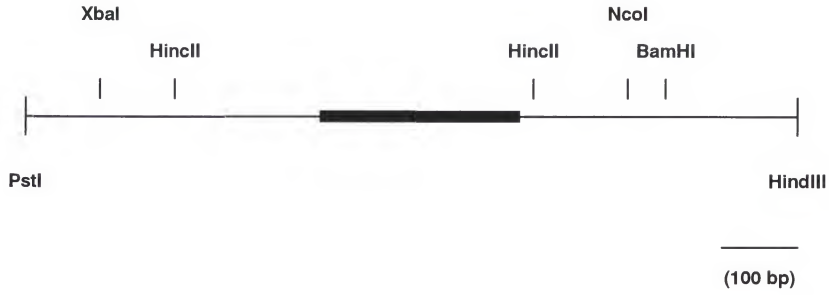


Figure 3-1. Restriction map of pAROB9-1 genomic fragment.

The thick black line indicates exon 9 of the porcine cytochrome P450 aromatase chromosomal gene.

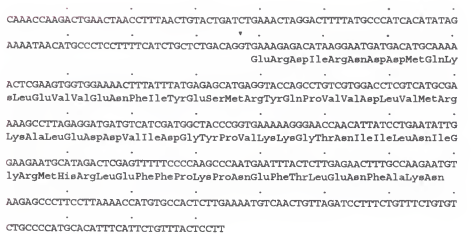


Figure 3-2. DNA sequence of exon 9 and exon-intron boundary sequences of the porcine aromatase gene.

The black arrow indicates the exon-intron splice junction and the predicted amino acid sequence of the encoded peptide sequence is shown below the DNA sequence.

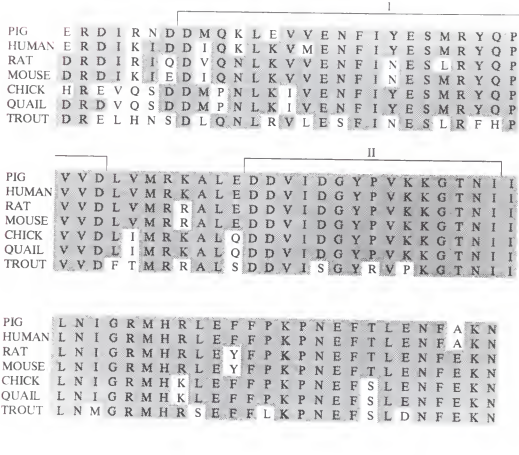


Figure 3-3. Comparison of the protein sequence encoded by pAROB9-1 with the corresponding regions of aromatase P450 proteins of six other vertebrate species.

The shaded region represents complete identity among the amino acid sequences. Regions I and II represent the ozols peptide (Corbin et al., 1988) and sequence of synthetic peptide used for antibody production, respectively.

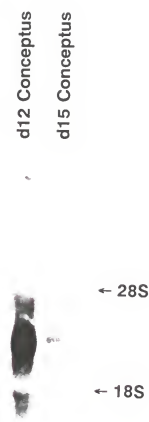


Figure 3-4. Relative abundance of aromatase P450 mRNA in developing porcine conceptuses.

A. Northern blot-hybridization analysis was carried out on total cellular RNA (30 ug) extracted from Days 12 and 15 conceptuses using the porcine antisense RNA as probe, as described under Materials and Methods.

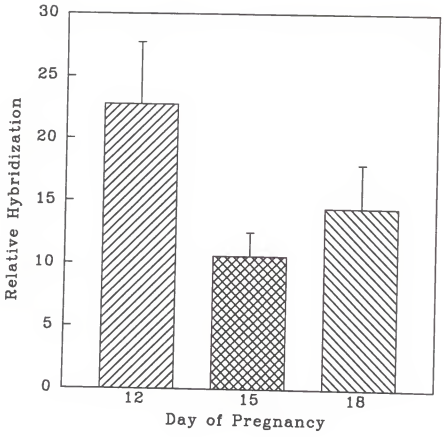


Figure 3-4. Relative abundance of aromatase P450 mRNA in developing porcine conceptuses.

B. Relative RNA levels were monitored by dot-blot hybridization of total RNAs from conceptuses at Days 12 (n = 4 pigs), 15 (n =3 pigs) and 18 (n = 4 pigs) of pregnancy followed by phosphorimage analysis (ICBR Core). The levels of aromatase P450 mRNA were higher at Day 12 (P = 0.07) than at Days 15 and 18.

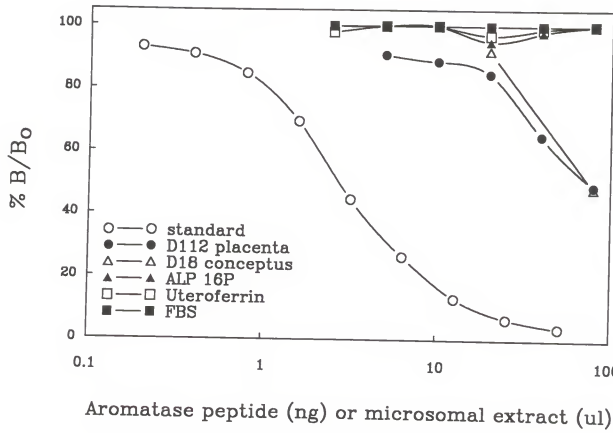


Figure 3-5. Validation of RIA for porcine aromatase cytochrome P450.

The specificity of binding of ^{125}I-aromatase oligopeptide (Arom 16P) to the antiserum was examined using increasing amounts of unlabelled Arom 16P and unrelated peptide (ALP 16P; Simmen et al., 1992), protein (uteroferrin) or fetal bovine serum (FBS). Increasing volumes of tissue extracts prepared from pig tissues were also used to displace the binding of labelled peptide to antiserum.

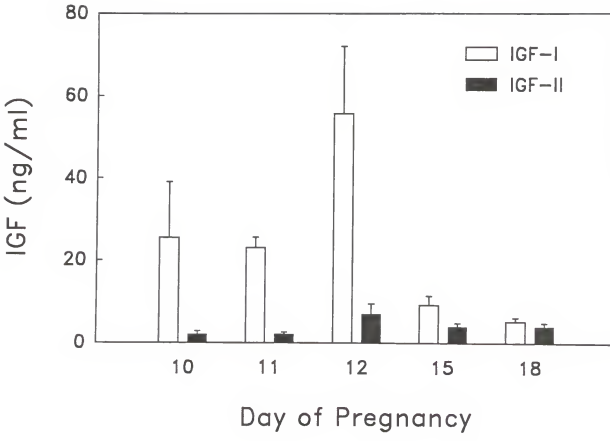


Figure 3-6. Amounts of IGF-I and IGF-II in pig uterine luminal fluids as a function of stage of early pregnancy.

Uterine luminal fluids were extracted in trifluoroacetic acid and chromatographed in Sep-Pak plus C_{18} columns to remove IGF binding proteins, prior to RIA as described under Materials and Methods. Values represent the mean ± SE of 6-7 independent samples.

Table 3-1. Correlation of conceptus aromatase P450 content and ratio of IGF-I to IGF-II concentrations in uterine luminal fluids (ULF) during the periimplantation period*

Pregnancy stage	Aromatase P450 (pg/μg DNA)	Total Aromatase P450 per Embryo (ng)	IGF-I:IGF-II
Day 10	6376.4[a]	6.38	12.3
Day 11	4784.9[b]	28.7	10.6
Day 12	1543.9[c]	15.4	7.9
Day 15	7.97[d]	0.37	2.3
Day 18	8.25[d]	1.07	1.3

* Values with different letters are significantly different from each other ($P < 0.01$)

Discussion

In the present study, the generation of molecular and immunological probes for porcine aromatase P450 mRNA and protein enabled the elucidation of the temporal relationships between the increased capacity for estrogen production by conceptuses and the uterine luminal fluid content of IGFs during late preimplantation embryo development. Results from these studies demonstrated that: 1) the levels of pig embryonic aromatase P450 mRNA and protein are temporally regulated during the late pre-implantation period; 2) the abundance of conceptus aromatase P450 protein parallels that of its corresponding mRNA as well as the capacity of conceptuses to secrete estrogens *in vivo*; and 3) the production of P450 aromatase protein in the conceptus parallels the ratio of immunoreactive IGF-I to IGF-II in the uterine microenvironment. Despite the known complexity of the transcriptional regulation of steroidogenic enzyme genes which are hormonally and developmentally modulated in a tissue-specific manner (Moore and Miller, 1991), the high degree of correlation between conceptus aromatase P450 production and uterine luminal fluid IGF concentrations *in vivo* is consistent with a role for this family of growth factors in an embryonic function critical to uterine receptivity and embryo implantation.

Several recent studies have explored the relationship between conceptus steroidogenic enzymes and estrogen production during the pre-implantation period in the pig. In the first study from our laboratories (Hofig et al., 1991), we demonstrated the preferential induction of aromatase P450 activity by IGF-I in Day 12 as opposed to Day 10 pre-implantation conceptuses *in vitro*. However, *in vitro* production of estrogens by these conceptuses was not correlated with the IGF-I induced aromatase activity, a finding

suggestive of the lack of additional regulatory or other factors in the culture system used which are involved in *de novo* steroidogenesis. In a second study from another group, Mason and colleagues (Conley et al., 1992) surveyed the expression of side-chain cleavage cytochrome P450, aromatase P450, 17α-hydroxylase cytochrome P450 (17α-P450) and 3ß-hydroxysteroid dehydrogenase in pig conceptuses during and after elongation, using the Western blot technique. They reported a positive correlation between levels of aromatase P450 and 17α-450 proteins with estrogen synthesis at the time of blastocyst elongation. The results of the present study, which utilized the more quantitative technique of radioimmunoassay, are consistent with those obtained in the previous study for aromatase P450. Additionally, Y. Ko in our laboratory localized the protein to the inner cell layer of the trophectoderm, in the hypoblast region which has been demonstrated to represent the site of estrogen synthesis in the porcine conceptus (Bate and King, 1988). Moreover, the parallel temporal changes in aromatase P450 mRNA and protein in this tissue suggest transcriptional control in the transient and cell type-specific expression of this steroidogenic enzyme during conceptus elongation.

The demonstration that the ratio of uterine luminal IGF-I to IGF-II, rather than concentrations of IGF-I or IGF-II alone, is positively correlated with levels of aromatase P450 mRNA and protein points to the possible concerted actions of these growth factors in regulating embryonic steroidogenesis. Indeed, trophectoderm of periimplantation pig conceptuses expresses IGF receptors (Corps et al., 1990; Green et al., 1995). The induction by IGF-I (Nestler, 1987) and the down-regulation by IGF-II (Nestler, 1990) of aromatase P450 activities in human placental cytotrophoblasts have been reported and may account for

this correlation for pig conceptuses. The concentrations of IGF-II were slightly increased by Day 12 when levels of IGF-I were maximal and which subsequently decreased thereafter. The signals for the near simultaneous changes in the levels of production and/or secretion of IGF-I and IGF-II around Day 12 have not been clearly defined; however, we have shown that estrogens from the conceptus, which are synthesized and secreted transiently beginning at Day 10 pre-implantation (Geisert et al., 1982a; Pusateri et al., 1990) can trigger the release of uterine-derived IGF-I (Simmen et al., 1990). The endometrial synthesis of IGF-I mRNA has been correlated with the increasing plasma levels of progesterone, whereas endometrial IGF-II mRNA levels do not change during the late pre-implantation period (Simmen et al., 1992a).

In addition to their effects on aromatase P450, IGF-I and IGF-II may also modulate embryonic steroidogenesis through the biosynthesis of the enzymes 17α-P450 and estrogen-2/4-hydroxylase. Estradiol concentrations in conceptuses were positively correlated with the levels of 17α-P450 mRNA and protein and with the progression of blastocyst development (Conley et al., 1992; Green et al., 1995). This positive correlation mimics that for aromatase P450 in the present study, where the levels of aromatase P450 mRNA and protein are high at Day 12 and then rapidly decline by Day 15, when blastocysts are filamentous in morphology. Similarly, the production of catechol estrogens by elongating pig blastocysts was correlated with a transient period of activity of the enzyme estrogen-2/4-hydroxylase (Mondschein et al., 1985). The expression of 17α-P450 is restricted in a tissue- and species-specific manner (Hum and Miller, 1993) and although the promoter and 5'-flanking region of the 17α-P450 gene have been demonstrated to exhibit functional activity in transient transfection studies (Brentano et al, 1990), potential sequences that may mediate growth

factor-induced gene transcription have not been defined. However, since sequences identified within the 5'flanking region of this gene exhibit similarities to those defined for the glucocorticoid responsive element, cAMP-responsive element and AP2 binding sites (Picado-Leonard and Miller, 1987), the potential involvement of the corresponding transcription factors, whose synthesis may be induced directly or indirectly by growth factors, appears likely. The ability of IGF-I to augment the luteinizing hormone-induced accumulation of 17α-P450 protein in ovarian theca-interstitial cells (Magoffin et al., 1990) suggests that the rapid decrease of this enzyme after Day 12 in porcine embryos may be correlated with the coincident decrease in secreted levels of IGF-I during this period (Letcher et al., 1989; this chapter). Similarly, although the potential for IGFs in regulating conceptus aromatase P450 biosynthesis is demonstrated in the present study, the mechanism of the transcriptional activation of the aromatase P450 gene by IGFs has not been elucidated. The human aromatase gene exhibits alternative promoter usage, one of which is regulated by protein kinase A and C pathways in human choriocarcinoma cells (Toda et al., 1990).

Finally, the direct role of conceptus-derived estrogens on the progression of conceptus development warrants further investigation. We have previously suggested that these estrogens, by modulating the synthesis and secretion of uterine-derived secretory products which are essential to development of conceptuses (Simmen and Simmen, 1990; Simmen et al., 1993; Simmen et al., 1995) may help support its timely progression into the post-implantation stage. One such secretory product is IGF-I, which acts as a mitogen for both the uterus (Simmen et al., 1988b) and the conceptus (Harvey and Kaye, 1992). The expression of a large number of growth factors which may be of uterine or embryonic origins

and whose biosynthesis is modulated by estrogens (Pollard, 1990; Simmen and Simmen, 1991), and the concerted actions of these growth factors (Paria and Dey, 1990) and their induced gene products may be required for coordinate maternal-embryonic development.

In summary, we have demonstrated the close temporal relationship between aromatase P450 synthesis by developing preimplantation porcine conceptuses and uterine luminal IGF content. The strong correlation between the levels of these regulatory molecules during the period of blastocyst elongation suggests a positive feed-back loop mechanism by which the levels of conceptus estrogens may transiently regulate and be regulated, by IGFs for the induction of embryonic and endometrial functions critical to embryonic development *in utero.*

CHAPTER 4
MOLECULAR CLONING OF AROMATASE CYTOCHROME P450 COMPLEMENTARY DEOXYRIBONUCLEIC ACID FROM PERIIMPLANTATION PORCINE AND EQUINE BLASTOCYSTS IDENTIFIES MULTIPLE NOVEL 5' UNTRANSLATED EXONS EXPRESSED IN EMBRYOS, ENDOMETRIUM, AND PLACENTA

Introduction

The gonads and the placenta during pregnancy constitute major endocrine sources of steroid hormones for reproductive processes. During early pregnancy, however, preimplantation blastocysts (or conceptuses) have also been identified as a significant autocrine/paracrine source of estrogens. Blastocysts from species as diverse as rat (Dey and Dickmann, 1974), hamster (Dickmann and Sen Gupta, 1974), rabbit (Hoversland et al., 1982; George and Wilson, 1978), pig (Perry et al., 1973; Gadsby et al., 1980), horse (Zavy et al., 1979), donkey (Heap et al., 1991), camel (Skidmore et al., 1994), and human (Edgar et al., 1993) produce estrogens in varying amounts. Although the role(s) of these estrogens is for the most part unclear, evidence suggests that they may be involved in the initiation of uterine changes associated with implantation (Dickmann et al., 1976; Bhatt and Bullock, 1974; Geisert et al., 1982; Simmen et al., 1990; Roberts et al., 1993). In this regard, the transient high level expression of blastocyst-derived estrogens in the pig is correlated with increased uterine endometrial gene expression and/or secretion of gene products important for embryo growth and nutrition as well as implantation (Roberts et al., 1993; Simmen et al., 1993; Simmen et al., 1995).

Another potential local source of estrogens during pregnancy is the endometrium. In the pig, the uterine endometrium exhibits no detectable aromatase activity during the estrous cycle or in a pseudopregnant state, maintains low levels of aromatase activity from early to midpregnancy, and achieves maximal aromatase activity in late pregnancy (Knight and Jeantet, 1991). Expression of the aromatase gene and its protein product in normal endometrium of cycling women is controversial, and studies concerning the possible expression of the aromatase gene in endometrium of pregnant women are lacking (Tseng et al., 1982; Huang et al., 1989; Taga et al., 1990; Bulun et al., 1993). The potential importance of studying endometrial aromatase gene expression during pregnancy when rapid uterine tissue growth is occurring, is underscored by the potential insights such studies may provide into the local effects of estrogens in the control of endometrial function associated with embryonic and feto-placental development.

Cytochrome P450 aromatase, the product of the CYP19 gene, is responsible for the conversion of androgens (C_{19}) to estrogens (C_{18}). Three successive hydroxylation reactions of C_{19} are catalyzed by the aromatase:NADPH-cytochrome P450 reductase complex with 3 molecules of O_2 and 3 molecules of NADPH used in the reaction (Thompson and Siiteri, 1974; Hanukoglu, 1992). Recent evidence suggests that P450 aromatase might also exhibit estrogen-2/4-hydroxylase activity (Osawa et al., 1993). This observation is consistent with the demonstrated parallel transient peak in expression levels of P450 aromatase and 17α-hydroxylase enzymes at the level of mRNA and protein (Conley et al., 1992; Ko et al., 1994; Conley et al., 1994; Green et al., 1995) with estrogen-2/4-hydroxylase activity (Mondschein et al., 1985) in periimplantation pig blastocysts. The molecular regulation of gene expression

as well as the structure of the P450 aromatase gene have been intensively investigated in the human. The human aromatase gene is comprised of 9 exons within a ~35 kb region that encodes the polypeptide (Means et al., 1989; Harada et al., 1990; Toda et al., 1990) and exhibits a number of tissue-specific and/or developmentally regulated 5' exons generated via alternative promoter usage and splicing mechanisms (Harada, 1992; Mahendroo et al., 1993; Harada et al., 1993; Toda et al., 1994; Honda et al., 1994). The most distal 5' exon is located more than 45 kb upstream of exon 2 (containing the ATG initiation codon) (Honda et al., 1994) and is the major 5' exon expressed in human placenta (Toda et al., 1990; Mahendroo et al., 1991). In contrast, the 5'-flanking region of exon 2 is the major promoter utilized in human ovary and corpus luteum (Means et al., 1991; Harada et al., 1993). Much less is known about the complexity of the aromatase gene 5'-terminus in other species. To date, two distinct 5' untranslated exons have been described for the bovine gene, whereas the chicken and Medaka fish aromatase genes appear to use only the promoter proximal to exon 2 (Hinshelwood et al., 1993; Vanselow and Furbaβ, 1995).

No studies reported to date have examined the nature of aromatase RNA transcripts in preimplantation embryos of any species. In the present study, the full-length cDNA and putative exon(s) 1 of porcine embryonic cytochrome P450 aromatase were isolated and characterized. In addition, the tissue and stage of pregnancy-dependent utilization of these sequences were examined as an initial step toward understanding the molecular mechanism(s) involved in P450 aromatase gene regulation in porcine periimplantation blastocysts and endometrium and placenta of pregnancy. Lastly, the corresponding 5' end of equine conceptus aromatase cDNA was cloned and sequenced and was found to be distinct from the

analogous porcine embryo sequences. The results suggest that the multiple 5' exon(s) of the aromatase chromosomal gene in mammals diverged much more rapidly than the body of the gene, which may reflect a unique mechanism(s) for species-specific regulation of this important steroidogenic enzyme gene in embryos and feto-maternal tissues during development.

Materials and Methods

Tissue Collection and RNA Extraction

Pigs were bred at estrus (Day 0) after at least two estrous cycles of normal duration (17-22 days) were observed. Animals on the indicated days of pregnancy were slaughtered at the Meats Processing Facility (University of Florida) and reproductive tracts were immediately removed and placed in ice. Embryos were collected by flushing the uterine lumen with phosphate-buffered saline (8g NaCl, 200 mg KCl, 1.44 g Na_2HPO_4, 240 mg K_2HPO_4/L, pH=7.4) and immediately subjected to RNA extraction. Uterine horns were opened longitudinally, and placental and endometrial tissues collected and stored at -80°C until used. Total cellular RNA was extracted using either TRIzol reagent (Life Technologies; Gaithersburg, MD) (blastocysts and conceptuses), the method of Puissant and Houdebine (Puissant and Houdebine, 1990) (ovary and endometrial tissues), or the standard guanidine thiocyanate/5.7M CsCl procedure (Sambrook et al., 1989) (placenta). Poly $(A)^+$-RNAs were purified using oligotex-dT affinity resin (Qiagen, Chatsworth, CA) and resuspended in 5mM Tris-HCl (pH 7.5). Total cellular RNA or poly $(A)^+$-RNA was quantified by absorbance at 260 nm.

Construction and Hybridization Screening of a Porcine Embryo cDNA Library

A cDNA library of Day 12 porcine conceptus mRNAs was constructed by use of ZAP-cDNA library synthesis and ZAP-cDNA Gigapack II Gold cloning kits (Stratagene, La Jolla, CA). Total cellular RNA was extracted from Day 12 blastocysts [92.5% filamentous and 7.5% spherical] collected from nine gilts. 4.5 μg poly $(A)^+$-RNA and oligo(dT) primers were used for the synthesis of 1st strand cDNA. Double stranded cDNA was synthesized and the size-fractionated RNAs (~1.5-4 kb) were used for linker ligation and subsequent cloning in the UNI-ZAP phagemid vector. After packaging of phagemids, the cDNA library was titered (866,000 independent clones) and amplified. Hybridization screening of approximately 300,000 phage clones was conducted using replica filters and a [^{32}P]-radiolabeled, 5'-*EcoRI* fragment of gel-purified human aromatase cDNA (Harada, 1988) which was nick-translated to a specific activity of about 1 X 10^8 cpm/μg (Amersham, Arlington Heights, IL). Eighty-one positive clones were obtained from the primary screening, and twelve independent clones were purified after secondary screening of eighteen positive clones. Hybridization was carried out in 6X SSC/5X Denhardt's solution/0.5% SDS/yeast RNA (250μg/ml) with 0.65 $\times$ 10^6 cpm/ml probe for 18 hours at 62°C. Filters were washed in 2X SSC/0.1% SDS for 30 minutes at room temperature and in 0.2X SSC/0.1% SDS for 1 hour at 62 °C before exposure to X-ray film. Bluescript phagemids containing cDNA inserts of the twelve purified clones were generated by *in vivo* excision.

DNA Sequencing and Restriction Enzyme Mapping

Double stranded DNA sequencing was performed using the Sequenase-based DNA sequencing kit (United States Biochemicals, Cleveland, OH). Restriction enzyme maps for the 33F and 34B clones were determined by use of the MAP command of the GCG (Genetics

Computer Group, University of Wisconsin) program and sites were confirmed by restriction endonuclease digestion. The DNA sequences reported in this chapter have GenBank accession numbers 3U37309, 5U37310, U37311, U37312 and U37313.

Northern and Southern Blot Analyses

Thirty μg of total cellular RNA from embryos and 5 μg of poly $(A)^+$-RNA purified from endometrial and placental tissues were electrophoresed in a 1.5% agarose/2.2 M formaldehyde gel and transferred to a Biotrans nylon membrane using the TurboBlotter (Schleicher and Schuell, Keene, NH). The membrane was hybridized in 6X SSC/2X Denhardt's solution/0.1% SDS, yeast RNA (250 μg/ml) with [^{32}P]-radiolabeled porcine aromatase cDNA probe (BamHI fragment from the 33F clone spanning exons 1A - 5) prepared by gel purification and nick translation. After washing the membrane once in 2X SSC/0.1% SDS for 20 minutes at room temperature and once in 0.2 X SSC/0.1% SDS for 20 min at 62°C, the membrane was exposed to X-ray film for two weeks at -80 °C. Southern blot analysis was carried out as described previously (Ko et al., 1994) using the hybridization conditions described above for cDNA library screening.

RT-PCR Analyses

Five μg of total cellular RNA or 1 μg of poly $(A)^+$-RNA were used for synthesis of 1st strand cDNA (cDNA Cycle kit for RT-PCR; Invitrogen, San Diego, CA). Polymerase chain reaction (1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C) was performed in a 50 μl volume containing 1X buffer (50 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 0.001% gelatin), 50 nmol dNTPs, 50 pmol of each primer, 1 unit of Taq polymerase (Boehringer Mannheim), and 2μl or 1μl aliquots of 1st strand cDNA synthesized from total

RNA or poly $(A)^+$-RNA, respectively, as template. Buffers A, B, C, D, F, and J are components of the PCR Optimizer kit (Invitrogen). Buffers A, B, C and D have the same pH (8.5) and contain 60 mM Tris-HCl and 25 mM $(NH_4)_2SO_4$, respectively but differ in the concentration of Mg^{2+} (A; 1.5, B; 2.0, C; 2.5, D; 3.5 [mM]). Buffers F and J contain 60 mM Tris-HCl, 25 mM $(NH_4)_2SO_4$ and 2.0 mM Mg^{2+} but differ in pH (F; 9.0, J; 9.5). Ten to 15 μl amplification product from each PCR reaction were electrophoresed in a 0.6% agarose gel. The sequences of each exon-specific oligonucleotide primer were as follows:

E1A, 5'-AGAGCAACTACTCGTGCGAAAGATC-3'

E1B, 5'-CAGAGACTTGGTACAGTCAAGACCC-3'

E2, 5'-GCATTATAAAGTCACCAGCATGGTG-3'

E3, 5'-CAGACTCTTATGAATTCTCCATACG-3'

E4, 5'-CTTCATTACATGGAACACAC-3'

E7, 5'-GGATTTGAAAGAGGACATGGAAATTCTG-3

E9, 5'-CTCAAGAGTAAATTCATTGGGCTTGGGG- 3

P3, 5'-CTGGTATTGAGGATGTTTCTTCATG- 3'

P1, same as E3

P2, same as E2

5'-RACE (Rapid Amplification of cDNA Ends)

5'-RACE was conducted using a Life Technologies kit (5'-RACE System). One μg of RNA isolated from Day 12 porcine embryos or Day 14 equine embryos was used as starting material. The E4 primer was used for priming of cDNA synthesis from both the

porcine and equine RNA preparations. RNA in a volume of 24 μl was used for the synthesis of 1st strand cDNA [final composition, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 100 nM primer, 16 mM dNTPs, and 8 units SuperScript II RT] for 30 minutes at 42°C. After removal of RNA with RNase H, the 1st strand cDNA was purified using a GlassMax DNA isolation spin cartridge (Life Technologies). An anchor sequence was then added to the 3' end of the cDNA using terminal deoxynucleotidyl transferase and dCTP and the final products were subjected to PCR amplification. PCR was conducted under the same conditions as described for RT-PCR analysis except that either an anchor primer (provided in kit) and the E3 primer (for porcine aromatase) or an equine aromatase-specific primer (5'-CTGGTATTGAGGATGTTTCTTCATG-3') designed from the sequenced equine exon 2 region were used. Amplified double stranded cDNA products were subcloned in PCR II and transformed into competent INVαF' cells (TA cloning kit, Invitrogen). The DNA sequence of each clone was analyzed using the BESTFIT command of the GCG program and the predicted junctions for exons 1, 2, and 3 were determined by comparison with the known intron-exon junctions for the human, chicken and Medaka fish aromatase chromosomal genes. The relative positions of the intron-exon junctions for exons 2 - 10 are perfectly conserved in all three species (Means et al., 1989; Harada et al., 1990; Toda et al., 1990; Matsumine et al., 1991; Tanaka et al., 1995).

Results

Isolation of cDNA Clones Encoding Porcine Cytochrome P450 Aromatase

A unidirectional, oligo(dT)-primed cDNA library was prepared using mRNA from porcine Day 12 embryos (mixture of spherical and filamentous blastocysts) and screened by hybridization with a radiolabeled 5'-terminal EcoRI fragment of human aromatase cDNA (Harada, 1988) for isolation of full-length cDNA clones. Eighty-one positive clones were identified from screening of 3×10^5 independent phagemid clones, and twelve of these were subsequently purified upon rescreening. The cDNA inserts of these twelve clones were subjected to further analysis by restriction endonuclease digestion and DNA sequencing of their 5'-ends using the T3 primer site of the vector. Among these twelve clones, only one (designated 34B) exhibited a distinct pattern of restriction endonuclease digestion due to a longer 3'-terminus. The 5' nucleotide sequences of all twelve clones were identical, where overlapping. Complete DNA sequencing was performed on the two clones that were identified as having the longest 5' (clone 33F) and 3' (clone 34B) ends, respectively (Figure 4-1).

The open reading frames of the 33F (2470 bp) and 34B (2588 bp) cDNA inserts encoded identical aromatase proteins of 503 amino acids. As mentioned above, their size differences resided at the 5' and 3' termini, with the 34B clone representing a mRNA that utilized a downstream polyadenylation site (Figures 4-1 & 2). Where overlapping, the DNA sequence of the 33F clone was identical to that of the porcine aromatase chromosomal exons 5 - 10 which were previously sequenced (Chapters 3 & 5; Choi and Simmen, 1994). The 34B clone, however, differed from this previous sequence at 8 nucleotide positions. Three of these differences were clustered at the end of the exon 5 region, with the remainder located in the 3' untranslated region of exon 10 (Figure 4-2).

The deduced amino acid sequence of pig embryo aromatase was compared with aromatase protein sequences of other species (Figure 4-3). This comparison identified regions of high and low homology. The putative transmembrane span of the porcine protein was hydrophobic as expected, but was not well conserved in sequence among aromatase proteins. In contrast, regions predicted to contribute to the aromatase active site (Chen and Zhou, 1992; Amarneh et al., 1993; Graham-Lorence et al., 1995) were generally well-conserved in human, porcine and bovine aromatase proteins, but were less conserved with those of rodents and lower vertebrates. These conserved regions included the β'-C loop, the I helix, the β1-4 and the β4 sheets (Figure 4-3) (Graham-Lorence et al., 1995). The heme-binding region containing the cysteine residue that serves as the fifth coordinating ligand for the heme group iron atom was also well conserved. Two consensus N-glycosylation sites (Asn-X-Ser/Thr) were identified in the amino-terminal region (Asn 43 and Asn 78) of the porcine aromatase protein. The first glycosylation site was present in an analogous position in the trout protein, whereas the second was present in an analogous position in the two avian aromatase proteins. Previous work indicated that aromatase activity may be regulated by phosphorylation mechanisms (Bellino and Holben, 1989). Consistent with this, a consensus sequence (KKGT) for phosphorylation by cAMP-dependent protein kinase within an extremely conserved domain located immediately downstream of the β1-4 region was observed (Figure 4-3). Computer-assisted analysis also located potential phosphorylation sites for casein kinase II (Thr 80, Ser 247 and Thr 462) and protein kinase C (Thr 113, Ser 247, Ser 363 and Ser 497), respectively.

Further comparisons of the porcine aromatase cDNA sequence with those from other species identified high sequence homologies downstream of the nucleotide at position 94, with no or very limited homology upstream of this nucleotide position. As this point of divergence is the exact location of the splice site for the multiple 1st exons and exon 2 of the human aromatase gene (Harada, 1992; Mahendroo et al., 1993; Harada et al., 1993; Toda et al., 1994; Honda et al., 1994), the sequence upstream of this point was designated as a putative exon 1 (E1A) of the pig aromatase gene.

Aromatase mRNAs in Porcine Embryos, Endometrium and Placenta

Northern analysis of embryonic total cellular RNA or endometrial and placental poly(A)$^+$-RNA was performed using a radiolabeled porcine aromatase cDNA fragment. Consistent with previous results (Chapter 3; Ko et al., 1994; Green et al., 1995), high level of expression of a ~2.7 kb aromatase mRNA was detected in spherical and filamentous Day 12 embryos. Previous studies from other groups (Gadsby et al., 1980; Fischer et al., 1985) identified aromatase activity in Day 18 conceptuses; however, we were unable to detect aromatase mRNA in Day 18 embryos, possibly due to the relatively low expression of this gene at this stage of embryo development. Aromatase mRNA was detected in placenta at midpregnancy (Day 60), but not at earlier days, even when poly(A)$^+$-RNA was used for the analysis.

Similarly, no expression of the aromatase gene was detected in porcine endometrium on Day 30 and Day 60 of pregnancy by Northern blot analysis (Figure 4-4). However, aromatase transcripts were identified in endometrial tissues by RT-PCR. In these studies, RT-PCR on total cellular RNA (0.5 ug) from endometrium of three animals for each of Days 30

and 60 of pregnancy using the E7 and E9 primer pair and 30 cycles of amplification yielded the expected PCR product in all samples tested (data not shown).

Evidence for Alternative Splicing of Pig Aromatase mRNAs

As described above, the human aromatase gene is characterized by a number of tissue-specific promoters and by alternative splicing of 5' untranslated exons. The regulation of expression of this gene in the human is controlled, in part, by sequences flanking these tissue-specific exons (Mahendroo et al., 1991; Mahendroo et al., 1993; Harada et al., 1993; Toda et al., 1992; Michael et al., 1995). By comparison, rat, chicken and Medaka fish aromatase chromosomal genes apparently lack a similar degree of complexity at their 5' ends. It was of interest therefore, to examine whether alternative splicing of aromatase mRNAs to yield different transcript sizes or 5' exons occurs in porcine embryos and feto-maternal tissues. The presence of the E1A sequence, the major putative 1st exon of aromatase mRNA in Day 12 embryos, was evaluated using RT-PCR. Initial assays were conducted using three different pairs of porcine aromatase-specific primers and 0.5 of RNA extracted from endometrium and placenta on Day 30 and Day 60 of pregnancy. Amplification for 30 cycles with exon 7 and exon 9 primers (E7-E9) as positive control, yielded the expected product for both tissue RNAs. In contrast, amplification with exon 2-exon 3 (E2-E3) and exon 1A-exon 3 (E1A-E3) primer pairs yielded products only from Day 30 placental total RNA (data not shown). The lack of E1A-E3 amplification in endometrial (Day 30 and Day 60) and Day 60 placental tissues could be due to alternative splicing which would yield different exons 1; however, there was no obvious explanation for the absence of the E2-E3 PCR product, especially as published evidence of alternative splicing of exon 2, which encodes the NH_2-

terminus of aromatase for any species, is lacking. In contrast, the expected PCR products were obtained for all three primer pairs using pig embryonic total cellular RNAs. Subsequently, poly(A)$^+$-RNA was isolated from endometrial and placental tissues to enrich for aromatase mRNAs. Using 50 ng poly(A)$^+$-RNA from endometrium or placenta and 500 ng total RNA from embryos, RT-PCR analysis was repeated following the conditions described above (Figure 4-5). Consistent with earlier results, PCR products using the three primer pairs were obtained for Day 12 embryo RNA. Interestingly, amplifications with E2-E3 and E1A-E3 primer pairs using Day 30 endometrial and placental tissue poly (A)$^+$-RNAs yielded the expected PCR products; however, these products remained absent in Day 60 endometrial and placental tissue poly (A)$^+$-RNAs.

Next, attempts were made to examine the possible effects of cycle number and buffer composition on the efficiency of amplification of E2-E3 and E1A-E3 products from Day 60 endometrium and placenta and as a positive control, from ovary tissue taken from pigs at periestrus (Figure 4-6). PCR amplifications used the same primer pairs, buffer conditions, and starting amounts of poly(A)$^+$-RNA as in previous experiments for 30 (lanes 1-3 for each panel) or 35 (lanes 4-6 for each panel) cycles. Additional PCR amplifications were conducted using E2-E3 (lanes 7-12 for each panel) or E1A-E3 (lanes 13-18 for each panel) primers under different buffer conditions (see Materials and Methods). In ovary, only the E7-E9 amplification product was detected after 30 cycles, whereas products from all three primer pairs were apparent after 35 cycles (Figure 4-6, top panel). There was no significant effect of buffer composition on the amplification of E2-E3 and E1A-E3 products. In Day 60 endometrium, E7-E9 and E2-E3 but not E1A-E3 amplification products were detected after

35 cycles of amplification under all buffer conditions (Figure 4-6, middle panel). PCR products from E7-E9, E2-E3 and E1A-E3 pairs were detected at 35 cycles for Day 60 placenta (Figure 4-6, bottom panel). Taken together, these results demonstrate the presence of aromatase polyadenylated mRNAs in porcine embryos, ovary, endometrium and placenta and suggest the relative underutilization or absence of exon 1A sequences in endometrium and placenta at midpregnancy.

Alternative 5'-Untranslated Aromatase Exons in Porcine Embryos

To examine more closely the possible heterogeneity of aromatase 5' cDNA sequences in Day 12 embryos, 5'-RACE was conducted using porcine embryo total cellular RNA. Two different classes of cDNA clones that share exon 2 and 3 regions (junctions defined by analogy to the human, chicken and Medaka fish genes) but have distinct 5' exons were identified by restriction enzyme analysis and DNA sequencing (Figure 4-7). Eighteen of nineteen 5'-RACE clones contained the E1A sequence; the longest among these had an additional 23 bp of 5' sequence relative to the original 33F cDNA clone. The remaining 5'-RACE clone had a distinct 189 bp sequence that did not exhibit any homology with aromatase 5'-untranslated exon sequences of other species; as such, this sequence may correspond to a putative second 5' exon (designated E1B). The putative exon sequences in these two classes of embryo cDNA clones diverged upstream of the nucleotide position (marked with an arrow (▾) in Figure 4-7) identical to the exon 1 - exon 2 splice site of the human aromatase gene.

Expression of E1B sequence-containing mRNA in Day 12 embryos was confirmed by RT-PCR coupled with Southern blot hybridization (Figure 4-8). The predicted PCR product from E1A-E3 but not from E1B-E3, primers was detected after 30 cycles of amplification.

Increasing the amplification cycles to 35 allowed for detection of the E1B-E3 PCR product, an observation consistent with a much lower frequency of aromatase mRNA transcripts containing the E1B sequence in Day 12 embryos. Likewise, the predominance of E1A-containing aromatase transcripts was found for Day 30 endometrium and placenta, whereas no or very low expression of the E1A-containing mRNAs was observed in Day 60 endometrium and Day 60 placenta (data not shown). The apparent lack of expression of E1A and E1B sequences in Day 60 endometrium and the lower expression of E1A and E1B sequences in Day 60 placenta relative to that in Day 30 placenta, suggests that yet another 5' untranslated exon(s) may be present in aromatase transcripts in these two tissues during the second half of gestation.

Cloning of Equine Aromatase cDNA

The horse is another species whose embryos secrete substantial amounts of estrogens during the preimplantation period (Zavy et al., 1979). Therefore, it was of interest to examine whether equine embryos utilized the same aromatase 5' exons (in particular, E1A), as porcine embryos. Partial cDNA clones that encode equine aromatase mRNA and that span the putative exons 1 - 3 were isolated from a pool of two Day 14 horse embryos by RT-PCR, followed by 5'-RACE (Figure 4-9). Two clones isolated by RT-PCR that contain exons 2 and 3 (by analogy to the human aromatase gene) had identical nucleotide sequences. The nucleotide sequences of three clones obtained by 5'-RACE were also identical, where overlapping. The deduced amino acid sequence of the amino-terminus of equine aromatase protein was homologous with the corresponding regions in pig (88%), cow (83%) and human (88%) aromatase proteins, respectively. Less homology was observed at other positions of

the available equine aromatase protein sequence with those of pig and human. Moreover, no homology was found among the putative exon 1 cDNA sequence of equine aromatase, the E1A or E1B sequences of porcine aromatase, or any other corresponding aromatase cDNA sequences of other species.

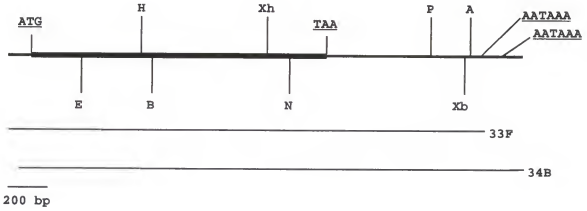


Figure 4-1. Restriction map of porcine cytochrome P450 aromatase cDNA clones.

Two porcine embryo P450 aromatase cDNA clones (33F and 34B) were sequenced in their entirety. The area encoding the P450 aromatase protein is shown as a thick black line with translational initiation (**ATG**) and stop (**TAA**) codons indicated. The first and second polyadenylation signal sequences (**AATAAA**) used for the 33F and 34B mRNAs, respectively, are shown. (A, AccI; B, BamHI; E, EcoRI; H, HindII; N, NcoI; P, PstI; Xb, XbaI; Xh, XhoI).

Figure 4-2. Nucleotide and amino acid sequences of porcine cytochrome P450 aromatase. The combined nucleotide sequences 1 - 2450 and 2451 - 2649 are derived from the 33F and 34B clones, respectively.

The two polyadenylation sequences are double underlined. The 33F clone contains a poly A tract after nucleotide 2450. Eight nucleotides with bold underlined letters in the coding sequence (**g**, **t**) and in the 3'-untranslated region (**t**, **a**, **t**, **a**, **c**) differed between the 34B clone and the 33F cDNA clone and previously sequenced genomic exons (Ko et al., 1994; Choi and Simmen, 1994). The first six nucleotides with bold underlined letters; "**g**, **t**, **t**, **t**, **a**, **t**" were "a, c, c, c, g, a" in the 34B clone, respectively. The "**a**" at 2460 was an additional nucleotide in the 34B clone and the "**c**" was a "t" in the genomic exon 10.

```
ctttacagagcaactactcgtgcgaaagatctaaaaactagcagaaaggattttctacag  60
agaaagatataaagaagggtcacaacaagacaggacttaaattgcttcctctgagatca  120

agcaatgcaagatggttttggaaatgctgaacccaatgcattataaagtcaccagcatgg  180
           MetValLeuGluMetLeuAsnProMetHisTyrLysValThrSerMetV
                .    .    .    .    .    .    .    .    .
tgtctgaagttgtgccttttgccagcattgcagtcctgctgctcactggctttcttctct  240
alSerGluValValProPheAlaSerIleAlaValLeuLeuLeuThrGlyPheLeuLeuL
                .    .    .    .    .    .    .    .    .
tggtttggaattataaaaacacatcttcaataccaggtcctggctattttctgggaattg  300
euValTrpAsnTyrLysAsnThrSerSerIleProGlyProGlyTyrPheLeuGlyIleG
                .    .    .    .    .    .    .    .    .
ggcccctaatttcctacctcagatttcctctggatgggcattggcagtgcctgcaactact  360
lyProLeuIleSerTyrLeuArgPheLeuTrpMetGlyIleGlySerAlaCysAsnTyrT
                .    .    .    .    .    .    .    .    .
acaacaaaacgtatggagaattcataagagtctggataggtggagaagaaacactcatta  420
yrAsnLysThrTyrGlyGluPheIleArgValTrpIleGlyGlyGluGluThrLeuIleI
                .    .    .    .    .    .    .    .    .
ttagcaagtcctcaagtgtgttccatgtaatgaagcatagtcactacacatcccgatttg  480
leSerLysSerSerSerValPheHisValMetLysHisSerHisTyrThrSerArgPheG
                .    .    .    .    .    .    .    .    .
gcagcaaacctgggttgcagttcattggcatgcatgagaaaggcattatattcaacaata  540
lySerLysProGlyLeuGlnPheIleGlyMetHisGluLysGlyIleIlePheAsnAsnA
                .    .    .    .    .    .    .    .    .
atccagtcctctggaaagctgttagaacttatttttatgaaagctctgtccggccctggcc  600
snProValLeuTrpLysAlaValArgThrTyrPheMetLysAlaLeuSerGlyProGlyL
                .    .    .    .    .    .    .    .    .
tggtgcgcatggtgaccgtctgtgccgattccatcaccaagcacctggacaagctggagg  660
euValArgMetValThrValCysAlaAspSerIleThrLysHisLeuAspLysLeuGluG
                .    .    .    .    .    .    .    .    .
aggtccgcaatgacttgggctacgtggacgtgttgaccctcatgcggcgcatcatgctgg  720
luValArgAsnAspLeuGlyTyrValAspValLeuThrLeuMetArgArgIleMetLeuA
                .    .    .    .    .    .    .    .    .
acacctctaacaacctcttcctgggatccgttgtggatgaaaaggccattgtgttaaaa  780
spThrSerAsnAsnLeuPheLeuGlyIleProLeuAspGluLysAlaIleValCysLysI
                .    .    .    .    .    .    .    .    .
tccagggttattttgatgcatggcaagctctccttctcaaaccagacatcttctttaaga  840
leGlnGlyTyrPheAspAlaTrpGlnAlaLeuLeuLeuLysProAspIlePhePheLysI
                .    .    .    .    .    .    .    .    .
ttccttggctgtacagaaagtatgaaaagtctgtaaaggatttgaaagaggacatggaaa  900
leProTrpLeuTyrArgLysTyrGluLysSerValLysAspLeuLysGluAspMetGluI
                .    .    .    .    .    .    .    .    .
ttctgatagaaaaaaaaagacgcaggattttcacagcagaaaaactggaagactgcatgg  960
leLeuIleGluLysLysArgArgArgIlePheThrAlaGluLysLeuGluAspCysMetA
                .    .    .    .    .    .    .    .    .
atttcgccactgagttgattttggctgagaaacgtggtgaactgacaaaagagaatgtga  1020
spPheAlaThrGluLeuIleLeuAlaGluLysArgGlyGluLeuThrLysGluAsnValA
                .    .    .    .    .    .    .    .    .
accagtgcatactggaaatgctaattgcagcaccagacaccatgtctgtcactgtgttct  1080
snGlnCysIleLeuGluMetLeuIleAlaAlaProAspThrMetSerValThrValPheP
                .    .    .    .    .    .    .    .    .
tcatgctgtttctcattgcaaagcacccccaggttgaagaggaactaatgaaggaaatcc  1140
heMetLeuPheLeuIleAlaLysHisProGlnValGluGluGluMetLysGluIleG
```

Figure 4-2 -- continued.

```
agactgttgttggtgaaagagacataaggaatgatgacatgcaaaaactcgaagtggtgg  1200
lnThrValValGlyGluArgAspIleArgAsnAspAspMetGlnLysLeuGluValValG

aaaactttatttatgagagcatgaggtaccagcctgtcgtggacctcgtcatgcgaaaag  1260
luAsnPheIleTyrGluSerMetArgTyrGlnProValAspLeuValMetArgLysA

ccttagaggatgatgtcatcgatggctacccggtgaaaaagggaaccaacattatcctga  1320
laLeuGluAspAspValIleAspGlyTyrProValLysLysGlyThrAsnIleIleLeuA

atattggaagaatgcatagactcgagttttccccaagcccaatgaatttactcttgagga  1380
snIleGlyArgMetHisArgLeuGluPhePheProLysProAsnGluPheThrLeuGluA

actttgccaagaatgttccttacaggtacttccagccatttggctttgggccccgggcct  1440
snPheAlaLysAsnValProTyrArgTyrPheGlnProPheGlyPheGlyProArgAlaC

gtgcgggaaagtatatcgccatggtcatgatgaaggtcactctggtcatacttctgagac  1500
ysAlaGlyLysTyrIleAlaMetValMetMetLysValThrLeuValIleLeuLeuArgA

gcttccaagtgcagacaccgcaagaccggtgtgttgaaaagatgcagaagaaaaatgatt  1560
rgPheGlnValGlnThrProGlnAspArgCysValGluLysMetGlnLysLysAsnAspL

tatccttgcacccggatgagaccagcggcctgctggaaatgattttcatcccaagaaatt  1620
euSerLeuHisProAspGluThrSerGlyLeuLeuGluMetIlePheIleProArgAsnS

cagacaagtgtttcactaaataaaattggtcagtccctgccctggaccagttctcaacag  1680
erAspLysCysPheThrLys

tattccacatggaaaccacccatctttgctagctaatcctcctcacatgaacattctgtg  1740
gcctattgtgttttatcagcttacctcctctgtgttatcagcataccagatgcactgttc  1800
tcctaagcatattcaagccagaaaccagactgcaaagaacatatggaggccaagagtttg  1860
tgcaagaaactatagccttaaaggaccatttccacaaaacaggttttggaaaaatggaga  1920
catcaacaaactcattctcatctcttctctgttctactgtgagaagggaacctttaatg  1980

tctggggcagaggcactcaagttgattagaaaggtccagctaacatctggatacctatgg  2040
ccaaacatacatggtaatttgatttggggtttggtgggatttggggactacaacatccga  2100
agccttgaagaaatgcttacaaattcagcatgtgactttttctataaattatattcaatt  2160
aacacttatttattcacatgtgatttatctgcagcaaaagttaaatcagagaatagactt  2220
gtggctgcctcaggggagaggaagggagtgggaggaatcgggagcttggggttaatggat  2280
gcaatctattgctcttggaatggatttacaatgagatcatgctgtgtagcattgagaact  2340
atgtctagatacttacatcgcaacacaacagtgggaggagaaaagtatgtatacatgtatg  2400
tgtaacttggtccccatgctgtacaacggaaataaatttaattaattaattaaaaaaaaaa  2460
gttacatcaaagagcatccttgcccaatgtctcaatttatgcctcaaccagaccatatct  2520
ctgctatgggggggaaaaaaatcacaaaatgtgtttcaaatttaaataaaattcttttttt  2580
atgttgcattgttaaaaaaaacaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  2640
aaaaaaaaa
```

Figure 4-3. Comparison of cytochrome P450 aromatase amino acid sequences.

The similarity of amino acid sequence of pig aromatase, determined by the BESTFIT command of the GCG package, relative to aromatase of other species was 94% for bovine (Hinshelwood et al., 1993; Vanselow and Furbaβ), 90% for human (HUM; Harada, 1988; Toda et al., 1989; Corbin et al., 1988), 88% for mouse (MOU; Terashima et al., 1991), 86% for rat (Hickey et al., 1990), 83% for chicken (CHI; McPhaul et al., 1988), 83% for zebra finch (ZEB; Shen et al., 1994), 72% for rainbow trout (TRO; Tanaka et al., 1992), and 71% for catfish (CAT; Trant, 1994), respectively. Identical amino acids are marked by dots and amino acid gaps that are deleted are indicated by dashes (-). Consensus glycosylation sites are identified by single underlines. TM is the putative transmembrane span (bold letters). I, II, III, HB and IV designate the β'-C loop, I helix, β1-4, heme-binding and β4 regions (Graham-Lorence et al., 1995), respectively. A well conserved, putative peptide sequence for phosphorylation by cAMP-dependent protein kinase is shown by bold underlined letters.

```
                                                    TM
PIG                        MVLEMLNPMH YKVTSMVSEV VPFASIAVLL LTGFLLLVWN  40
COW                        .L..V...R. .N..... ..I....I.. ..........
HUM                        ........I. .NI..I.P.A M.A.TMP... ...LF.....
MOU                        .F.......Q .N..I..P.T .TVSAMPL.. IM.L...I..
RAT                        .F........ .N..I..P.T ..VSAMPL.. IM.L...IR.
CHI                        .IP.T...LN .-F..L.PDL M.V.TVPIII .IC..F.I..
ZEB                        ....T..L.. .NI..L.PDT M.V.TVPI.I .MC..F.I..
TRO  MDLLSPVCG RVMAVVCLDT VIADL.VSES RNA.ATR..G ISL.TGSL.. .LCL..AA.-
CAT  MAAHVFPMCE RTRKPVHFSE T.M.I.LREA RNG.DPRY.N PRGITLLL.. CLVL..T...

PIG -YKNTSSIPG PGYFLGIGPL ISYLRFLWMG IGSACNYYNK TYGEFIRVWI GGEETLIISK  99
COW -.ED..... .S..... ..HC..... ..... M....M...V C.........
HUM -.EG..... ...CM..... ..HG..... .....R V....M.... S.........
MOU -CESS..... ...C..... ..HG..... ..... M....M.... S.........
RAT -CESS..... ...C..... ..HG..... ..... M....M.... S.........
CHI -HEE..... ...CM..... ..HG..... V.N..... .....V.... S....F....
ZEB -HEE..... ...CM..... ..HG..... V.N..... ...D.V.... S....F....
TRO RHTDNN.V.. .FFC..V... L.....I.T. ..T.S....S K..DIV.... N....F.L.S
CAT RHEKKC.... .SFC..L... M..C..I... ..T.S....E K..DMV.... S.....VL.R
                                    I
PIG SSSVFHVMKH SHYTSRFGSK PGLQFIGMHE KGLIFNNNPV LWKAVRTYFM KALSGPGLVR 159
COW ...M..... ...I..... L..... .....A ......PF.T .........
HUM ...M..I... N..S..... L...C..... .....E ...TT.PF.. .........
MOU ...M..... ...I..... R...C..... N.....S ..RTI.PF.. ...T.....
RAT ...MV..... .N.I..... R...C..... N.....S ..RT..PF.. ...T....I.
CHI ......... WN.V..... L...C...Y. N.....A H..EI.PF.T .........
ZEB ....S..... W..V..... L...C...Y. N.....A H..EI.PF.T .........
TRO ..A.H..LRQ GR..... Q..SC...D. R.....S.MA ...KT...A ...T....QK
CAT P.A.Y..L.. .Q..... L...C..... Q.....S.VT ..RK....A ...T....Q.

PIG MVTVCADSIT KHLDKLEE-V RNDLGYVDVL TLMRRIMLDT SNNLFLGIPL DEKAIVCKIQ 218
COW ...I..... ....R...-. C..... ..... ..M..... ..S...VN..
HUM ......E.LK T...R...-. T.ES..... ..L..V..... ..T...R... ..S...V...
MOU ..E..VE..K Q...R.G.-. TDTS..... ....H..... ..M..... ..S...K...
RAT ..E..VE..K Q...R.GD-. TDNS.....V ....H..... ..T..... ..SS..K...
CHI .IAI.VE.TI V.....-. TTEV.N.N.. N..... ..K....V.. ..S...L...
ZEB .IAI.VE.T. E...R.Q.-. TTE..NINA. N..... ..K....V.. ..N...L...
TRO T.D..VS.TQ T...A.QGPD GLMG.Q.... S.L.CTVV.I ..R....V.. N..ELLQ...
CAT TLEI.TM.TN T...G.-SRL TDAQ.H.... N.L.C.VV.I ..R...DV.. N.QNLLF..H

PIG GYFDAWQALL LKPDIFFKIP WLYRKYEKSV KDLKEDMEIL IEKKRRRIFT AEKLEDCMDF 278
COW ..... .....S ..C..... ....DA.... .AE..H..S. .....SI..
HUM ..... I.....S ...K..... ....DAI.V. .AE.....S. E....E....
MOU ...N..... I..N.....S .....R.. ....DEIAV. V....HKVS. .....
RAT ...N..... I..N.....S .....R.. ....DEI... V....QKVSS .....
CHI N..... .....S ..CK...EAA ....GA.... ..Q..QKLS. V...DEH...
ZEB N..... .....S ..CK..KDA. ....GA.... ..Q..QKLS. V...DEH...
TRO K...T..TV. I...VY..LD .IHE.HRRAA QE.HDAI.D. VDQ...GLQE .D..DH-IN.
CAT R..ET..TV. I...FY.RLK ..HD.HRNAA QE.HDAI.D. ..Q..TELQQ ....DN-LN.
```

Figure 4-3 -- continued.

```
                                        II
PIG ATELILAEKR GELTKENVNQ CILEMLIAAP DTMSVTVEFM LFLIAKHPQV EEELMKEIQT  338
COW .....F.... ....R..... .......... .....S.... .......... ..AIIR....
HUM .......... .D..R..... .......... .....SL... ........N. ..AII.....
MOU ..D..F..R. .D........ .......... ......LY.. .L.V.EY.E. .AAIL...H.
RAT ..D..F..R. .D........ .......... ......LYV. .L...EY.E. .TAIL...H.
CHI .SQ..F.QN. .D..A..... .V...M.... ..L...L.I. .I...DD.T. ..KM.R..E.
ZEB .SQ..F.QN. .D..A..... .V...M.... ..L...L... .I...E..T. ...M.R..E.
TRO TAD..F.QSH ..ISA...R. .V...V.... ..L.ISL... .L.LKQN.D. .LQ.LE..D.
CAT TE...F.QSH ....A...R. .V...V.... ..L.IS.... .L.LKQNAE. .RRILT..H.
                                        III
PIG VVGERDIRND DMQKLEVVEN FIYESMRYQP VVDLVMRKAL EDDVIDGYPV KKGTNIILNI  398
COW ........I. .....K.... ..N..... .......... .......... .........L
HUM .I.....KI. .I...K.M.. .......... .......... .......... ..........
MOU ...D...KIE .I.N.K.... ..N..... .......R.. .......... ..........
RAT ...D....IG .V.N.K.... ..N..L.... .......R.. .......... ..........
CHI .M.D.EVQS. ..PN.KI... .......... ....I..... Q......... ..........
ZEB ...D...QS. ..PN.KI... .......... ....I..... Q......... ..........
TRO AI.D.ELH.S .L.N.R.I.S ..N..L.FH. ...FT..R.. S....S..R. P........M
CAT .L.DTELQHS HLSQ.H.L.C ..N.AL.FH. ...FSY.R.. D....E.FR. PR.......V
                                        HB
PIG GRMHRLEFFP KPNEFTLENF AKNVPYRYFQ PFGFGPRACA GKYIAMVMMK VTLVILLRRF  458
COW .......... .......... .......... .......G+. .......... .V..T.....
HUM .......... .......... .......... .......G+. .......... AI..T.....
MOU .......Y.. .......... E......... .......G+. .......... .V..T.....
RAT .......Y.. .......... E......... .......S+. .......... .V..T..K..
CHI ....K..... .....S.... E....S.... .......G+V ..F....... AI..T....C
ZEB ....K..... .....S.... E....S.... .......S+V ..F....... AI..T....C
TRO .....S...L .....S.D.. E..I.N.F.. ...S...S+V ..H....... SI..T..S.Y
CAT .....S..Y. ..AD.S.D.. N.P..S.F.. ...S...S+V ..H....... AV.LMV.S..
                IV
PIG QVQTPQDRCV EEMQKENDLS LHPDETS-GL LEMIFIPRNS DKCFTK                  503
COW H...L.G... .......... ......R.DR ....T.... ...LER
HUM H.K.L.GQ.. .SI..IH... ......K.NM ....T..S. .R.LEH
MOU ..K.L.K..I .NIP..... ...N.DR.H. V.I..S.... .YLQQ
RAT H.K.L.K..I .N.P.N.... ..L..D..PI V.I..RHIFN TPFLQCLYIS L
CHI R...MKG.GL NNI..N.... M..I.RQ.P. ...V.T-QEA QTRIRVTKVD QH
ZEB R...MKG.GL NNI..N.... M..I.RQ.P. ...V.T..RN ANENQGDGMD QH
TRO S.CPHEGLTL DCLPQT.N.. QQ.V.EEGEP HT.K.L..HQ ARKQS
CAT S.CPEESCT. .NIAHT.... QQ.V.DK.HT .SVR.....T HTRNR.A
```

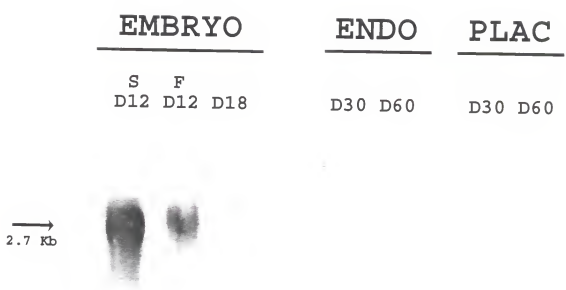


Figure 4-4. Northern blot analysis of P450 aromatase gene expression in porcine embryos, endometrium and placenta during early and midpregnancy.

Thirty μg of total cellular RNA from Day 12 blastocysts and Day 18 conceptuses and 5 μg poly(A)$^+$-RNA from the endometrial (ENDO) and placental (PLAC) tissues on the indicated days of pregnancy were electrophoresed. [^{32}P] radiolabeled porcine aromatase cDNA probe spanning exons 1A - 5 was used as a probe. The size of aromatase mRNA was determined by relative migration position to 28S and 18S rRNAs. S and F indicate spherical and filamentous forms of day 12 embryos, respectively.

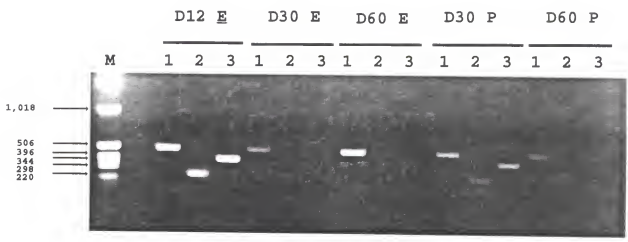


Figure 4-5. RT-PCR analysis of P450 aromatase mRNA in porcine embryos, endometrium, and placenta during pregnancy.

The presence of P450 aromatase mRNA in day 12 embryos (E), and day 30 and day 60 endometrium (E) and placenta (P) of pregnancy was examined by RT-PCR using three different pairs of primers (lanes 1, exons 7 and 9; lanes 2, exons 2 and 3; lanes 3, exons 1A and 3) and 30 cycles of amplification. The predicted sizes of the PCR products from exons 7-9, exons 2-3, and exons 1A-3 primer pairs are 502, 237, and 388 bp, respectively. The sizes (bp) of the DNA standards are indicated on the left.

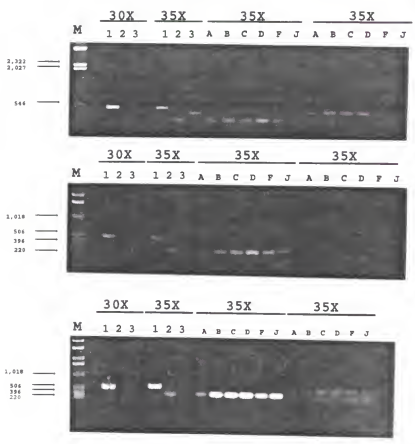


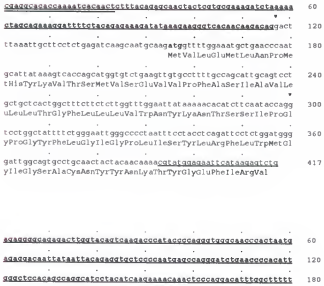
Figure 4-6. RT-PCR analysis of exon 1A-containing mRNA in porcine ovary, endometrium, and placenta.

PCR amplifications were carried out for ovary (Day 0) RNA **(top)**, Day 60 endometrial RNA **(middle)** and Day 60 placental RNA **(bottom)** using three primer pairs (lanes 1, exons 7-9; lanes 2, exons 2-3; lanes 3, exons 1A-3) at 30 or 35, cycles. PCR amplifications of exon 2 - 3 (left-most set of lanes labeled A-J for each panel) and exon 1A - 3 (right-most set of lanes labeled A-J for each panel) were also conducted at 35 cycles in six buffers differing in $Mg2^{+}$ concentration (A - D) or pH (F and J). The numbers on the left side indicate the sizes (bp) of the DNA standards.

Figure 4-7. DNA sequences of two distinct types of embryonic aromatase cDNA clones isolated by the 5'-RACE procedure.

Two distinct porcine P450 aromatase cDNA clones with unique 5'-terminal DNA sequences were isolated from Day 12 pig embryos using the 5'-RACE procedure (see Materials and Methods). The DNA sequences of the putative first exons for the two clones denoted exon 1A (E1A, **A**) and exon 1B (E1B, **B**) are indicated by bold and underlined letters. The DNA sequence of exon 1A is identical to that of the 33F cDNA except for the additional 23 bp of 5' sequence (with the double underline). DNA sequences of exons 1A and 1B are distinct from each other and did not have any homology with the known 5' untranslated exons of cytochrome P450 aromatase genes (cDNAs) of other species. The point of divergence of the sequences of the two clones (3' to 5' direction) (and the position of a splice junction in the human aromatase gene) is indicated by the first arrow. The second arrow indicates the position of predicted exon 2-exon 3 junction. The 3' primer is underlined.

A.



B.

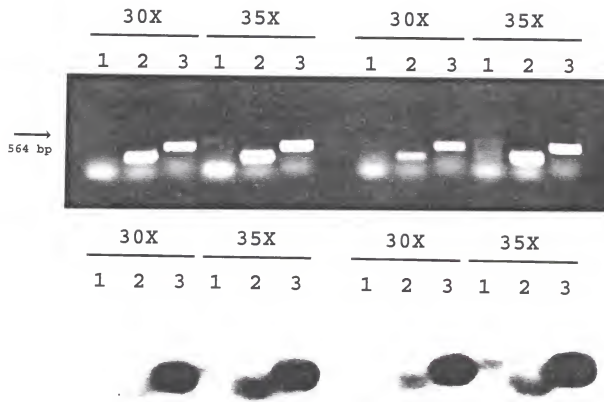


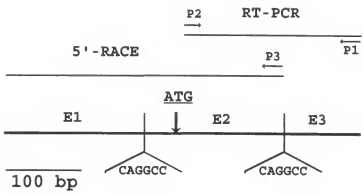
Figure 4-8. Detection by RT-PCR of exon 1B transcripts in Day 12 porcine embryos.

Upper Panel. RT-PCR was performed with three different pairs of primers (lanes 1, exons 1B-3; lanes 2, exons 2-3; lanes 3, exons 1A-3) at 30 (30X) or 35, (35X) cycles of amplification with spherical embryos (left half) or filamentous embryos (right half).
Lower Panel. Amplification of exon 1B transcript was confirmed by Southern hybridization with [^{32}P] radiolabeled porcine P450 aromatase cDNA previously used for Northern analysis. The DNA in the gel shown in the upper panel was transferred to a nylon membrane and the resultant autoradiogram from the hybridization analysis is shown. The expected product size for the exons 1B-3 primer pair is 483 bp.

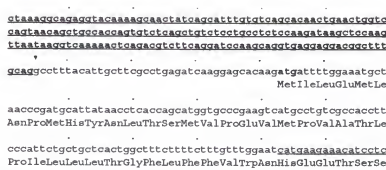
Figure 4-9. Cloning strategy and 5' cDNA and amino acid sequences of equine embryonic P450 aromatase.

A. cDNA for equine P450 aromatase was synthesized by RT-PCR using primers specific for porcine P450 aromatase (P1, exon 3; P2, exon 2; see Materials and Methods for the primer sequences) and total RNA previously extracted and pooled from two equine day 14 embryos. Amplified equine P450 aromatase cDNA was subcloned and subjected to DNA sequencing. For the 5'-RACE procedure, P1 was used to synthesize the 1st strand cDNA and P3 which was designed from the cDNA sequence of the equine RT-PCR clones was used for PCR amplification with a 5' anchor primer. The RT-PCR clones and the 5'-RACE clones are overlapping at the putative equine exon 2 (E2) region and collectively cover portions of the putative exons 1 (E1) through 3 (E3). DNA sequences of each putative exon boundary (CAGGCC) and the location of translation initiation codon (ATG) are indicated.
B. cDNA and deduced amino acid sequences of equine P450 aromatase covering putative exons 1-3. Bold letters with underline represent DNA sequences of putative E1 and underlined letters represent the DNA sequence of primer P3 used for the 5'-RACE procedure. The presumptive splice junctions for E1, 2 and 3 are indicated by arrows.

A.



B.


ctaaaggcagaggttacaaaagcaactatcaggatttgtgtcagcacaactgaactggtca 60
cagtaacagctggcagcagtgtctcagctgtctcctgcctctccaagataagctccaagg 120
ttaataaggtcaaaaactcagacgtcttcaggatccaagcaggtgaggagcaccggctttg 180
gcaggcctttacattgcttcgcctgagatcaaggagcacaagatgattttggaaatgcta 240
MetIleLeuGluMetLeu
aacccgatgcattataacctcaccagcatggtgcccgaagtcatgcctgtcgccaccttg 300
AsnProMetHisTyrAsnLeuThrSerMetValProGluValMetProValAlaThrLeu
cccattctgctgctcactggctttcttttctttgtttggaatcatgaagaaacatcctca 360
ProIleLeuLeuLeuThrGlyPheLeuPhePheValTrpAsnHisGluGluThrSerSer
ataccaggccctggctattgcatgggaatcgggccctcatttcacctccggttcctgtg 420
IleProGlyProGlyTyrCysMetGlyIleGlyProLeuIleSerProProValProVal
gatggggcttggcagtgcctgcaactactacaacaaga 458
AspGlyAlaTrpGlnCysLeuGlnLeuLeuGlnGln

Discussion

In this report, we describe the isolation of several distinct classes of P450 aromatase cDNA clones from late preimplantation stage pig blastocysts that differ in lengths of their 3' untranslated region and in their nucleotide sequences at the 5' ends. These clones appear to be derived by differential usage of polyadenylation signals and by alternative splicing of unique 5' exons. Unlike the two, more widely spaced, polyadenylation signals found in human and mouse aromatase mRNAs (Means et al., 1989; Toda et al., 1989; Terashima et al., 1991), the corresponding polyadenylation signal motifs in porcine aromatase mRNAs are only separated by 127 nucleotides and do not yield mRNAs that are easily resolved on standard Northern gels. Sequence heterogeneity in the 5' ends of the porcine embryonic aromatase mRNAs identified untranslated E1A and E1B sequences lacking homology with any of the known human, bovine or rat aromatase 5'-untranslated exons. Based on the corroborative results from library screening, 5'-RACE and RT-PCR analyses, our studies demonstrate that mRNAs containing the E1A sequence and using the upstream polyadenylation signal constitute the major aromatase transcript in Day 12 pig embryos, with embryo mRNAs containing the E1B sequence or using the downstream polyadenylation signal occurring at a much lower frequency.

Porcine aromatase, like those from human, bovine and rodent, is comprised of 503 amino acids. Immunoblot assays previously identified either a single aromatase protein band (M_r of 49 kDa) or three aromatase protein bands (M_rs of 45-48 kDa) in periimplantation porcine blastocysts and/or conceptuses (Conley et al., 1992; Ko et al., 1994). Our sequence

analysis of two independent porcine cDNA clones with complete open reading frames revealed identical amino acid sequences for a protein with a calculated M_r of 58 kDa. These results are consistent with the molecular weight variants being derived from differences in extent of glycosylation or proteolysis rather than in protein sequence. In this regard, we noted two potential glycosylation sites just downstream of the predicted transmembrane span in the pig aromatase sequence. Human placental aromatase is glycosylated at one site; however, this site (Asn 12), which is located upstream of the predicted transmembrane region and on the opposite side from the protein's functional domains (Shimozawa et al., 1993), is absent in an analogous position in the porcine protein. This appears to be a real difference between human and porcine aromatase and not a consequence of potential mutations introduced during cDNA cloning or a sequencing error, because the Asn → Lys substitution at position 12 (human vs. pig) was observed in both full-length porcine cDNA clones and in fifteen 5'-RACE cDNA clones sequenced across this region. Interestingly, although chicken aromatase also lacked this consensus glycosylation site at Asn 12, equine aromatase, like the human protein, contains this sequence motif. The biological ramifications, if any, of these differences in potential glycosylation sites remain to be determined.

Recent evidence from enzyme activity and substrate utilization studies indirectly suggests significant phylogenetic differences in the three-dimensional structures of the P450 aromatase catalytic domain (Amri et al., 1993; Swinny et al., 1993). Homology comparisons of full-length aromatase proteins from five mammalian, two avian and two piscine species highlighted the regions in which significant sequence changes have occurred. In particular, these regions include the amino-terminus, membrane-spanning region, carboxy-terminus, and

β4 sheet structure near the carboxy-terminus, that was previously modeled to overlay the A ring of substrate (Graham-Lorence et al., 1995). Protein regions that are highly conserved include the I helix and heme-binding domains that also contribute to the substrate-binding region, and the domain that lies just downstream of the β4 sheet structure (Graham-Lorence, 1995). Within the latter, we identified a well conserved, consensus cAMP-dependent protein kinase phosphorylation sequence that was localized to the protein surface by molecular modeling and mutational analysis (Amarneh et al., 1993; Graham-Lorence et al., 1995). The phosphorylation of this consensus site in vivo to regulate aromatase activity is an interesting possibility.

The nature of the regulatory mechanism(s) which underly the transiently high expression of P450 aromatase in pig embryos during the preimplantation period remains unclear. The work described here raises the possibility that this transient expression may be related to novel, relatively embryo-specific 5' exons (E1A and E1B) in porcine aromatase cDNAs, not otherwise observed for 5' exons in aromatase cDNAs of other species. However, equine periimplantation embryos, which like their porcine counterparts secrete large amounts of estrogens, did not exhibit a 5'-untranslated sequence homologous to those identified for the porcine species. As we cannot completely rule out the possibility that alternative 5' exons do not exist for equine embryo cDNAs due to the rather limited number of equine 5' cDNA clones examined in this study, the E1A sequence that represents the major 5' exon utilized in the porcine embryo may in actuality define a species-conserved 5' exon. These results are consistent with our findings that pig placental aromatase transcripts contain the E1A sequence, albeit at levels much lower than in pig embryos.

The tissue-specific expression of distinct 5'-untranslated exons has been reported for the bovine aromatase gene (Hinshelwood et al., 1993; Hinshelwood and Simpson, 1994; Vanselow and Furbaβ, 1995). Results from these studies demonstrated that bovine ovarian and placental aromatase mRNAs differ in their 5'-untranslated exon sequences. Whether such tissue specific differences in 5'-untranslated regions may account in part for the differential expression of aromatase in different species remains speculative. In pig placenta during early (Day 30) and mid pregnancy (Day 60), aromatase transcripts contain either the E1A sequence or other, as yet uncharacterized, 5' exons that may or may not be related to the currently known placental aromatase 5' sequences. On the other hand, only endometrial aromatase mRNAs from early (Day 30) but not midpregnancy (Day 60) stages exhibited the E1A sequence, although expression of the aromatase gene is probably higher on Day 60 than on Day 30. These latter results are consistent with stage of pregnancy-dependent changes in aromatase gene promoter usage and add another level of complexity to aromatase gene regulation in female reproductive tract tissues.

Previous workers identified uterine aromatase activity in pregnant pigs but not in nonpregnant or pseudopregnant pigs (Zavy et al., 1980; Fischer et al., 1985; Knight and Jeantet, 1991). Similarly, aromatase activity was demonstrated in endometria of pregnant rabbits during the periimplantation period, but no such activity was found in nonpregnant rabbits (Tseng et al., 1988). The reported synthesis of aromatase protein and mRNA in normal human uterine tissues is more controversial. Whereas several studies demonstrated aromatase activity and/or immunoreactive protein in cycling human endometrium (Tseng et al., 1982; Huang et al., 1989; Taga et al., 1990), no aromatase gene transcripts could be

detected in this tissue by use of the RT-PCR method (Bulun et al., 1993). Results from the present study demonstrate that pregnant pig endometrial tissues express aromatase mRNA, albeit at lower levels than in placenta, during midpregnancy. The lack of detectable endometrial aromatase mRNA expression as monitored by Northern analysis in the present study is consistent with previous observations from other laboratories which suggested that aromatase activity in this tissue as well as placenta peaks later than the time period studied here (Knight and Jeantet, 1991). In this regard, Ko et al. (1994) reported comparable levels of immunoreactive aromatase protein in Day 90 porcine endometrium and placenta. In work not shown here, our attempts to identify aromatase transcripts in endometrium from early pregnant pigs (Days 10, 12 and 18) by RT-PCR assays have not provided any evidence of gene expression during this time period. These data suggest that endometrial expression of the aromatase gene is activated or up-regulated at some time after initiation of implantation. This conclusion is supported by the results of Fischer et al. (1985), indicating the local induction of porcine endometrial aromatase activity by conceptuses.

The pig uterus exhibits a pronounced linear increase in weight throughout pregnancy, reflecting the marked growth occurring in this tissue to accommodate the rapidly increasing mass of fetuses and placentae. The results of our RT-PCR analyses indirectly suggest the presence of additional, as yet unknown, aromatase 5' exons in porcine endometrium and/or placenta at midpregnancy. However, we cannot rule out the possibility that these results were due to a less efficient amplification from E1A & E3 and E1B & E3 primer sets when using the RNA samples from midpregnancy as a template. Efforts to obtain cDNA clone(s) and corresponding sequences from midpregnancy endometrium are necessary in order to resolve

this issue (chapter 5). In light of the possible local growth-regulatory and/or differentiative actions of uterine-produced estrogens, further investigation of aromatase mRNA and protein expression in specific cell types of the uterus and as a function of stage of pregnancy are warranted. Moreover, it will be interesting to compare the sequences and transcriptional regulation of the aromatase gene promoters that function in porcine endometrium during pregnancy with those that drive aromatase gene expression in human neoplastic endometrium, a second example of a highly proliferative uterine state in which aromatase gene expression is well documented (Bulun et al., 1994).

CHAPTER 5
MOLECULAR CLONING OF MULTIPLE FORMS OF CYTOCHROME P450 AROMATASE AND THEIR DEVELOPMENTAL EXPRESSION IN PORCINE BLASTOCYSTS, ENDOMETRIUM, AND PLACENTA

Introduction

The pig is a species whose embryos (blastocysts) produce large amounts of estrogens during the periimplantation period (Days 11 and 12). These estrogens play an important role in regulation of uterine gene expression and protein secretion essential for embryo growth, survival, and implantation (Geisert et al., 1982; Pope et al., 1988; Roberts et al., 1993; Simmen et al., 1993; Simmen et al., 1995). There has accumulated a substantial body of biochemical (Perry et al., 1973; Hofig et al., 1991), immunohistochemical (Conley et al., 1992; Ko et al., 1994), and molecular biological (Ko et al., 1994 and Chapter 5) data demonstrating the developmental regulation of steroidogenic enzyme gene activity in periimplantation porcine embryos.

Estrone (E1) is known to be a major estrogen synthesized by periimplantation porcine embryos (Perry et al., 1973; Gadsby et al., 1980). However, there exists other unknown phenolic compounds produced by these same embryos whose biological functions have never been investigated (Gadsby et al., 1980; Fischer et al., 1985). Porcine blastocysts also synthesize and secrete large amounts of catechol estrogens (E_2-2/4-OH) (Mondschein et al., 1985) and 15α-hydroxy-estradiol-17β (15α-OH-E_2) (Chakraborty et al., 1990) during the

periimplantation period and in very similar temporal fashion to that of estrogens. Moreover, the recent identification of cytochrome P450 aromatase as the human placental estrogen-2/4-hydroxylase (Osawa et al., 1993) suggests additional possible steroidogenic products of porcine blastocyst aromatase such as the catechol estrogens. Catechol estrogen is known to be involved in the implantation process of some experimental animal models (Hoversland et al., 1982; Dey et al., 1986), via stimulation of prostaglandin synthesis (Kennedy, 1977; Kelly and Abel, 1980; Pakraski and Dey, 1983), which in turn may increase the permeability of endometrial capillaries. However, the physiological significance of this finding has not been investigated in the pig. In addition to its presence in pig blastocysts, catechol estrogen activity as well as aromatase activity has been demonstrated in preimplantation hamster blastocysts (Sholl et al., 1983), supporting the idea that an additional activity of aromatase in blastocysts is that of a catechol estrogen synthetase.

The demonstration of multiple catalytic activities of *in vitro* expressed human placental aromatase (Corbin et al., 1988) and identification of aromatase cDNA encoding a single isoform of aromatase from several species suggests that the three hydroxylation steps for the formation of the aromatic A ring of estrogens (Thompson and Siiteri, 1974) from different substrates are catalyzed by a single enzyme. On the other hand, the possible tissue- and/or species-specific activity of aromatase enzyme was indicated by other studies (Swinney et al., 1993). Recently, two protein sequences for isoforms of porcine aromatase in ovary and term placenta were reported (Corbin et al., 1995).

It has been demonstrated that aromatase activity in blastocysts including those of the human is a relatively common phenomenon, yet little information is available regarding the

molecular mechanisms involved in blastocyst expression of this gene. We have hypothesized that the presence of embryo-specific transcription factor(s) may be involved in the temporal regulation of aromatase gene expression in pig embryos. Therefore, as an initial step toward understanding the molecular mechanisms underlying the transient induction of aromatase gene expression in porcine blastocysts, two full-length cDNA clones (33F and 34B clones) were isolated from a cDNA library of Day 12 porcine embryos (Chapter 4). These two cDNA clones encoded identical aromatase protein sequences but differed in size due to alternative polyadenylation signal usage for the corresponding mRNAs. The deduced amino acid sequence of the embryo aromatase had 92 % identity to term placental aromatase, which was found to exhibit higher enzymatic activity than ovarian aromatase (Corbin et al., 1995). This suggests that embryo aromatase enzyme may have unique or additional catalytic activity such as the synthesis of unknown phenolic compounds, including catechol estrogens.

Two classes of cDNA clones that contained distinct (putative) exon 1 sequences (E1A and E1B, respectively) of porcine aromatase mRNA and which had no significant homology with the aromatase exon 1 sequences from other species reported to date, were also isolated from Day 12 porcine embryos (Chapter 4). This provided the initial evidence for alternative splicing of novel 1st exons(s) in the aromatase gene of preimplantation pig embryos, as was previously demonstrated for the tissue-specific expression of the human aromatase gene (Harada, 1992; Harada et al., 1993; Mahendroo et al., 1993; Honda et al., 1994; Toda et al., 1994). The demonstrated lack of homology of the two porcine embryo aromatase 5' untranslated exons and the equine blastocyst aromatase 5' untranslated exon (Chapter 4) may indicate that there are not only tissue and developmental-specific first exons but also species-

specific first exons for this gene. In addition, the results of RT-PCR presented in the previous experiment (Chapter 4) suggested the presence of aromatase transcripts containing additional 5' exons different from E1A and E1B, as well as of differences in coding region sequences, in endometrium and/or placenta during midpregnancy (Day 60). Therefore, the following experiment was performed to clone the putative aromatase isoform-encoding mRNA(s) favored in endometrium and placenta at midpregnancy, which would provide an additional clue(s) regarding the molecular basis for the developmental expression of this gene.

Materials and Methods

Tissue collection and RNA extraction

The same conceptus, endometrial and placental tissues and RNA preparations used in the previous experiment (Materials and Methods in Chapter 4) were utilized for this study .

RT-PCR cloning

Five μg of total cellular RNA or 1 μg of poly $(A)^+$-RNA was used for the synthesis of 1st strand cDNA (cDNA Cycle Kit for RT-PCR; Invitrogen, San Diego, CA). Two step polymerase chain reaction (30 seconds at 94°C, 40 seconds at 65°C, and 3 minutes at 72°C for ten cycles; and 30 seconds at 94°C, 40 seconds at 65°C, and 3 minutes and an additional 20 seconds in each subsequent cycle at 72°C for 20 cycles) was performed in a 50 μl volume containing 1X buffer [50 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin], 50 nmol dNTPs, 50 pmol of each primer, 2.6 units of Taq DNA polymerase and Pwo DNA

polymerase mix (Boehringer Mannheim), and 2μl or 1μl aliquots of 1st strand cDNA synthesized from total RNA or poly $(A)^+$-RNA, respectively, as template. A pair of primers designed from exon regions 2 and 10 of porcine embryo aromatase cDNA (see Figure 5-2) was used for the amplification. PCR products were subcloned in PCR II vector and transformed into competent INVαF' cells (TA cloning kit, Invitrogen). The remainder of the PCR product was digested with EcoRI endonuclease after purification (Wizard kit, Promega, WI) and was then subjected to agarose gel-electrophoresis.

DNA sequencing

Double-stranded DNA sequencing was performed using the Sequenase-based DNA sequencing kit (United States Biochemicals, Cleveland, OH) .

Southern blot analysis

Southern blot analysis was carried out as described in Chapter 4 (see Materials and Methods).

RT-PCR analyses

Five μg of total cellular RNA or 1 μg of poly $(A)^+$-RNA was used for synthesis of 1st strand cDNA (cDNA Cycle Kit for RT-PCR; Invitrogen, San Diego, CA). Polymerase chain reaction (1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C) was performed in a 50 μl volume containing 1X buffer [50 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin], 50 nmol dNTPs, 50 pmol of each primer, 1 unit of Taq polymerase (Boehringer Mannheim), and 2μl or 1μl aliquots of 1st strand cDNA synthesized from total RNA or poly $(A)^+$-RNA, respectively, as template. Ten to 15 μl of amplification product from each PCR reaction was electrophoresed in 0.8 % (Figure 5-1, 4 , and 5) or 2.0 %

(Figure 5-7) agarose gels.

Computer Analyses

The GCG program (University of Wisconsin, WI) was used for sequence alignment (PILEUP command), determination of homology (BESTFIT command), and hydropathy analysis (PEPPLOT command). The evolutionary tree was constructed using the MEGA (Molecular Evolutionary Genetics Analysis) software package (Kumar et al., 1993).

Results

RT-PCR cloning was performed using primers designed from the 33F clone sequence and mRNA isolated from Day 60 pregnant pig placenta as a template. To obtain cDNA clones encoding the entire porcine aromatase protein, two primers derived from immediately upstream of the translational initiation codon and immediately downstream of the stop codon, respectively and a mixture of Taq DNA polymerase and Pwo DNA polymerase were used for high fidelity PCR (Materials and Methods). The amplified, double-stranded DNA was ligated into plasmid vector, and used to transform into *E. coli* and ten randomly selected clones were subjected to EcoRI enzyme digestion. A different pattern of DNA bands was detected for the clones isolated from Day 60 placenta as compared to the 33F clone after electrophoresis. Whereas both the 33F and 34B clones isolated from Day 12 embryos (Chapter 4) had a single EcoRI site in the exon 3 sequence similar to human aromatase cDNA (Harada 1988), the cDNA clones isolated from Day 60 placenta exhibited three fragments after digestion with EcoRI enzyme (data not shown). These results

indicated that the DNA sequences of the placental clones might differ from those of the embryo 33F and 34B clones. Indeed, subsequent results obtained from partial DNA sequencing (exon regions 2 and 3) of one (P2 clone) of the ten clones indicated differences in nucleotide sequence from the 33F clone.

The same experimental approach was used to clone collections of pig aromatase cDNAs from the total RNA of Day 12 embryos, or the poly $(A)^+$-RNA isolated from Day 30 endometrium and placenta, Day 60 endometrium and placenta, and Day 0 ovary, respectively. In each case, the PCR products were subjected to purification, EcoRI digestion and agarose gel electrophoresis. Interestingly, whereas the EcoRI-digested amplification products from Day 12 embryos and Day 30 endometrium and placenta showed two major DNA fragments like the 33F clone, the EcoRI-digested PCR products from Day 60 endometrium and placenta consisted of three DNA fragments of two of which were different sizes (Figure 5-1). These results again suggested the possibility of expression of distinct types of aromatase mRNAs with different nucleotide sequences in the Day 30 vs. Day 60 endometrial/placental tissues.

To confirm the occurrence of a different cDNA sequence, the exon 2 and 3 regions of a total of forty-four cDNA clones isolated as described above were subjected to DNA sequencing. The mRNA transcript represented by the 33F cDNA clone was mainly expressed in Day 12 embryos consistent with the previous results and in Day 30 endometrium and placenta, whereas the other mRNA transcript represented by the P2 clone was the major transcript in Day 60 placenta and endometrium (Table 5-1). Complete DNA sequencing of nine different randomly selected clones, (three cDNA clones each from Day

12 embryos, Day 60 endometrium, and Day 60 placenta, respectively), was carried out and seventy-seven nucleotide differences (about 92 % identity) between the 33F and P2 clones were found. It should be noted that there were one or two nucleotide differences, on the average, found among the three cDNA clones from Day 12 embryos and among the six cDNA clones isolated from Day 60 endometrium and placenta, presumably representing PCR or sequencing errors or allelic polymorphisms. The P2 clone displayed an additional EcoRI site in the exon 6 region, which caused the altered pattern of DNA fragments after EcoRI digestion of PCR amplification products from Day 60 placenta and endometrium. This clone also had an insertion of four nucleotides (**AGCC**) slightly upstream of the stop codon, causing a frame shift of the open reading frame in the carboxyterminus of the encoded protein. (Figure 5-2).

An additional type of porcine aromatase cDNA (A10 clone) was isolated from Day 12 embryos (Figure 5-2 & 3). This clone, which was deleted in exons 4-6 at the exact exon-intron splice junctions of the human aromatase gene, contained an identical nucleotide sequence to that of the 33F clone except for one nucleotide change in exon 7, and maintained an open reading frame for a 354 amino acid aromatase related protein. It seems likely that this internal deletion of three exons resulted from an alternative RNA splicing mechanism. Interestingly, as the last two nucleotides (AA) of exon 3 and the first nucleotide (G) of exon 4 in the 33F clone constitute a codon for Lysine (AAG → K^{99}, #1 in Figure 5-3), the same last two nucleotides (AA) of exon 3 and the first nucleotide (A) of exon 7 in A10 clone constitute a codon for the same amino acid (AAA → K^{99}, #2 in Figure 5-3). It should be noted that one (E9 clone) out of nine midpregnancy endometrial clones was deleted in exon 3, which caused

a frame shift and consequently, a prematurely terminated protein in exon 4.

Comparisons of the deduced amino acid sequences of porcine cytochrome P450 aromatase proteins expressed in Day 12 embryos, Day 60 endometrium and placenta, and term placenta and ovary (Corbin et al., 1995) showed that only the exon 5 encoded polypeptide region was identical for all the clones although the peptides encoded by exons 9 and 10 are highly conserved (Figure 5-3). The putative peptide sequence for phosphorylation by cAMP-dependent protein kinase (**KKGT**) and the heme-binding domain (**FGPRACAGKYIAMV**) in exons 9 and 10, respectively, are conserved for all five aromatase isoforms. The first consensus glycosylation site (**KTS**) is conserved among four isoforms (#1-#4), whereas the second (**KKT**) is shared only by the 33F and A10 clones.

A high degree of similarity between the proteins encoded by the Day 60 and term placenta cDNA clones was noted. There exists only eight amino acid differences between the protein sequences encoded by the P2 clone (#3) and the term placenta cDNA clone (#4) (Figure 5-3). As indicated in Figure 5-2, there are five nucleotides in the P2 clone ($\underline{G}^{416}$, $\underline{T}^{724}$, $\underline{C}^{1311}$, $\underline{T}^{1387}$, and $\underline{T}^{1532}$, respectively) that were altered among the six cDNA clones which were isolated from Day 60 endometrium and placenta and sequenced in their entirety. The P2 clone contained a $\underline{G}^{416}$ instead of an A^{416} as in the other four clones and two clones (including the P2 clone) contained $\underline{T}^{1532}$ instead of the G^{1532} in four other clones. The $\underline{T}^{724}$, $\underline{C}^{1311}$, and $\underline{T}^{1387}$ were C^{724}, T^{1311}, and C^{1387}, respectively, in one of the six clones. The differences at positions 416 and 1532 may have resulted from incorporation errors by DNA polymerase. A change from $\underline{G}^{416}$ to A^{416} does not alter the amino acid used (K^{130} in Figure 5-3) but the change from $\underline{T}^{1532}$ to G^{1532} alters the amino acid used ($GA\underline{T}^{1532}$; D^{502} →

GAG[1532]; E[502]). Thus, it is likely that the actual amino acid at position 502 is "E", which is also found in the placental and ovarian aromatase isoforms, instead of "D". This indicates that there are only seven amino acid differences between the two placental aromatase sequences. It should be noted that these seven amino acid differences between the two placental aromatase sequences are not the result of the four nucleotide changes (G^{416}, T^{724}, C^{1311}, and T^{1387}, respectively) described above. Overall, the protein encoded by the 33F clone (#1) had more similarity with the placental proteins (#3; P2 and #4) than the ovary protein (#5) (Figure 5-3).

In a previous RT-PCR study (Chapter 4), amplification for 30 cycles with exon 7 and exon 9 primers (E7-E9) designed from the 33F clone sequence yielded the expected products for both endometrium and placenta at both pregnancy stages (Days 30 and 60), whereas no amplification products using either exon 1A and exon 3 (E1A-E3) or exon 2 and 3 (E2-E3) primer pairs were detected in Day 60 endometrium and placenta. Only after amplification for 35 cycles were products for Day 60 placenta detected; but no amplification was obvious for Day 60 endometrium (see Figure 4-5). As shown above, the P2 clone represents the major form of aromatase transcript expressed in Day 60 endometrium and placenta, but not in Day 12 embryos or Day 30 endometrium and placenta. As indicated in Figure 5-2, there are three nucleotides differences between the 33F clone (#1) and the P2 clone (#3) in the exon 2 (P1) and exon 3 (P2) primer sequences, which may explain why no amplification products were detected with these primers using RNA from Day 60 endometrium and placenta. Therefore, an attempt was made to determine whether this absence of amplification with E1A-E3 primers and E2 - E3 primers (previous chapter) was actually due to expression

of aromatase transcripts of the P2 clone type or to a less efficient amplification of 5' ends of cDNA from the Day 60 placental tissue.

A pair of primers was designed in exons 2 (P3) and 3 (P4) regions where the nucleotide sequences of the 33F and P2 clones were identical. RT-PCR was performed as described above (Materials and Methods) on mRNA isolated from Day 60 placenta and using four different primer pairs (lane 1, P1-P2; lane 2, P3-P4; lane 3, E1A-P4; and lane 4, E1B-P4, see Figure 5-4) for 30 cycles. Amplification product was apparent only for the P3-P4 primers. This result confirms that the lack of amplification products using E1A-E3 and E2-E3 primers in Day 60 placenta is not due to a less efficient PCR amplification of 5' end sequences but to less or no expression of the 33F clone prototype aromatase mRNA. In addition, this indicates a) that neither E1A nor E1B are the major forms of first exons in aromatase transcripts expressed in mid-pregnancy placenta, and b) a third type of first exon(s) may be expressed in this tissue and in endometrium at mid-/late pregnancy.

One (i.e., A10 clone) out of ten PCR-generated cDNA clones isolated from Day 12 porcine embryos was deleted in exon 4-6 sequences (Table 5-1). Previously, it was shown that the 33F clone represented the major aromatase mRNA expressed in Day 12 embryos (see Chapter 4). An experiment was conducted to confirm the presence of : a) the aromatase transcript deleted in exons 4-6, and b) P2 clone type transcripts in Day 12 embryos by using RT-PCR procedures. Four pairs of primers were designed from different exons as indicated in Figure 5-2. Only one primer (P7, Figure 5-2) was specific for P2 transcripts because of four nucleotide differences between the 33F and the P2 clones at the corresponding positions of this primer; whereas, the nucleotide sequences of all other primers

were identical between the two clones. As expected, amplification product was detected using the P5-P6 primer pair specific for the 33F clone, but there was no amplification product obtained using the P5-P7 primer pair specific for the P2 clone (Figure 5-5A). This again confirmed that the mRNA transcripts represented by the 33F clone sequence are the majors form of aromatase mRNA expressed in Day 12 embryos and suggests that P2 clone type transcripts are very rarely or not expressed in developing embryos. Two amplification products, of the expected sizes for the 33F and the A10 clones (P8-P6 primers, 653bp for the 33F clone and 296 bp for the A10 clone, respectively) were also apparent; however, no detectable amplification product was observed using P8-P7 primers (Figure 5-5A). Identity of the exons 4-6 deleted transcript in Day 12 embryos was verified by Southern blot analysis using a radiolabeled cDNA probe spanning exons 5-7 of the 33F clone (Figure 5-5B). Although no attempt was made to quantitate abundance of the transcripts represented by the 33F and A10 clones, it is apparent that the A10 clone transcript is significantly expressed in Day 12 embryos (Figure 5-5A).

The 5' untranslated cDNA sequence of porcine aromatase mRNA in Day 12 embryos (Figure 4-7A) was compared with that for porcine aromatase mRNA in term placenta (Hinshelwood et al., 1995) (Figure 5-6A). Interestingly, the 5' untranslated aromatase exon of term placenta is highly homologous (84% identity) to that of Day 12 embryo mRNA. This indicates that the nucleotide sequence of the term placental aromatase is highly homologous to that of the 33F clone not only in protein coding sequence, but also in 5' untranslated exon sequences. Therefore, it was interesting to speculate that there may be common embryo-specific aromatase isoforms for mammalian species. The horse is another

species whose embryos produce large amounts of estrogens during the periimplantation period. Complementary DNA clones containing the 5' end and coding exons 2-3 of aromatase transcripts expressed in Day 14 equine embryos previously were sequenced and high embryo expression of aromatase mRNA was confirmed by RT-PCR (Figure 4-9). An additional 5' untranslated cDNA sequence of equine aromatase cloned from term placenta was recently reported (Hinshelwood et al., 1995). As shown in Figure 5-6B, some similarity exists between the two 5' end cDNA sequences of equine aromatase mRNA, where overlapping. However, significant differences were also found. In particular, there was a fifty-seven nucleotide gap upstream of the putative exon 1/exon 2 splice junction and four base pair mismatches when the two sequences were compared (Figure 5-6B). Therefore, it was interesting to test the possibility that developmental expression of distinct 5' untranslated exons might occur in equine periimplantation embryos vs. placentae. Two pairs of primers, one downstream primer designed at the end of exon 2 and common for both sequences and two different upstream primers specific for embryo (P9) or term placenta (P10), and total RNA of Day 14 equine embryos were used for RT-PCR analysis. An amplification product of the expected size for the term placenta aromatase transcript, was detected using P10 and exon 2 primers (Figure 5-7). On the other hand, two amplification products, the larger band of the expected size (309 bp) for embryos using P9 and exon 2 primers and the smaller band of the expected size for the term placental transcript using P9 and exon 2 primers were observed. These results indicate that both equine placental and embryo 5' untranslated sequences are present in Day 14 equine embryos and that the actual nucleotide sequence of term placental aromatase, where the P9 primer was designed, may be identical to embryonic aromatase.

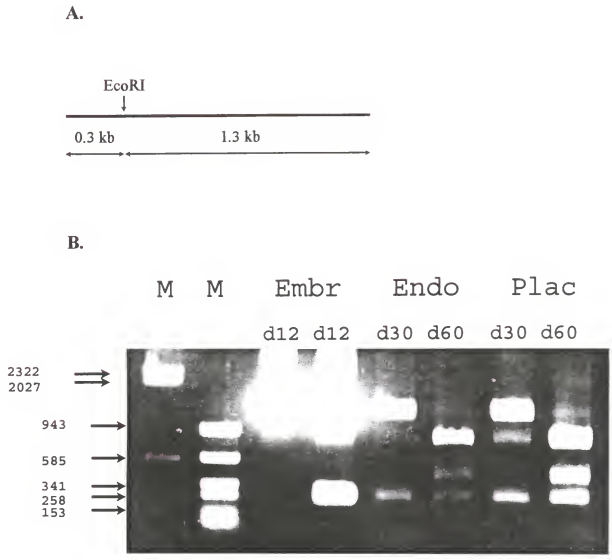


Figure 5-1. Developmental switch in cytochrome P450 aromatase isoforms type mRNA expression.

A. The expected location of the EcoRI site and sizes for the EcoRI-digested fragments of the PCR products based upon the structure of the 33F cDNA clone. **B.** Full-length aromatase cDNAs were generated by RT-PCR for the different tissues (Embr, embryo; Endo, endometrium; Plac, placenta) as described in Materials and Methods. Products, except for the left lane for Day 12 embryos (uncut), were digested with EcoRI after purification and prior to electrophoresis. The number on the left-hand indicate the sizes (bp) of the DNA standards.

Table 5-1. Summary of restriction endonuclease sites and sequence analyses of porcine aromatase cDNA clones obtained from six tissues.

Tissue Source	33F clone type	P2 clone type	Total
Day 12 Embryos	10	0	10*
Day 30 Placenta	6	1	7
Day 60 Placenta	0	10	10
Day 30 Endometrium	6	1	7
Day 60 Endometrium	0	9	9**
Day 0 Ovaries	1	0	1

A total of forty-four full-length cDNA clones (generated by RT-PCR and cloned in PCRII) were analyzed by restriction endonuclease digestion and DNA sequencing. Three clones each for the Day 12 embryo, Day 60 endometrium, and Day 60 placenta were sequenced in their entirety and a minimum of the exon 2 and 3 regions were sequenced for all the remaining cDNA clones. * Exons 4 to 6 of one clone (A10 clone) were found to be deleted. ** Exon 3 of one clone (E9 clone) was found to be deleted.

Table 5-2. Amino acid and nucleotide sequence identities of porcine cytochrome P450 aromatase isoforms.

	#1	#3	#4	#5
#1	100 (100)	92.6 (95.2)	92.4	88.2
#3	92.6 (95.2)	100 (100)	98.4	87.0
#4	92.4	98.4	100	87.6
#5	88.2	87	87.6	100

The identities of amino acids and nucleotides (in parentheses) of aromatase proteins encoded by cDNAs identified here (#1, 33F clone; #3, P2 clone) and by others (Corbin et al., 1995) (#4, from placenta and #5, from ovary), were determined by use of the BESTFIT command of the GCG program.

Figure 5-2. Sequences of porcine cytochrome P450 aromatase cDNAs encoding variant aromatase proteins.

Nucleotide sequences of two additional porcine P450 aromatase cDNAs (#2, A10 clone; #3, P2 clone, respectively), which were obtained by RT- PCR from Day 12 embryos (#2, 1133 bp) and placenta of mid-pregnancy (#3, 1584 bp), were compared with that of the 33F clone (#1) (Fig. 1A. in Chapter 4) using the PILEUP command of the GCG program. Identical nucleotides are marked by dots and nucleotide gaps are indicated by dashes (-). The thirty nucleotides of 5'- most sequence (CT...GG) and 3'- most sequence (CC...TC) are the primers used for PCR amplification to obtain full-length cDNAs that were cloned. The first nucleotide of each exon (by analogy to the human aromatase gene) is indicated by a number. The nucleotide sequences of the translational initiation codon (ATG), stop codons (TAA), and EcoRI site (GAATTC) common for three cDNA clones and additional EcoRI site for the #3 clone are shown by bold underlined letters, respectively. The nucleotide sequences of primers (P1 through P8) used for subsequent RT-PCR analysis are double underlined and differences in nucleotide identity among PCR-generated clones which were sequenced in their entirety (embryo, n=3; placenta, n=3; endometrium, n=3) are indicated by underlined letters. The deleted nucleotide sequence corresponding to exons 4 to 6 (#2, A10 clone) and the insertion of four nucleotides in the P2 clone are highlighted by shadowed dashes (-).

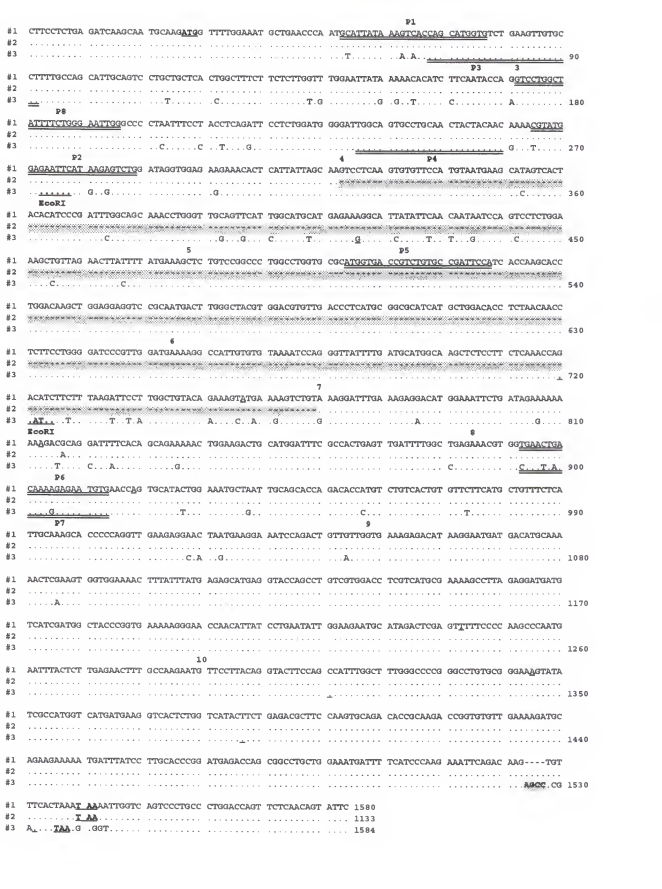

P1
P3
3
P8
P2
4
P4
EcoRI
5
P5
6
7
8
P6
P7
9
10

Figure 5-3. Alignment of porcine cytochrome P450 aromatase isoform-specific amino acid sequences.

The amino acid sequences of five different aromatase isoforms identified here (#1, 33F; #2, A10; and #3, P2 clone; respectively) and by others (#4, from placenta and #5, from ovary) (Corbin et al., 1995) were aligned by use of the PILEUP command of the GCG program. Identical amino acids are marked by dots and amino acid gaps are indicated by dashes (-). Consensus glycosylation sequences (**NTS** and **NKT**), a well-conserved, putative peptide sequence for phosphorylation by cAMP-dependent protein kinase (**KKGT**), and the heme-binding domain (**FGPRACAGKYIAMV**) are highlighted by bolded, underlined letters. The first amino acid of each exon (by analogy to the human aromatase gene) is marked by a number. The deleted amino acids encoded by exon 2 of the #5 clone and exons 4 to 6 of the #2 clone are highlighted by shadowed dashes (-).

```
                                                                                3
#1  MVLEMLNPMH YKVTSMVSEV VPFASIAVLL LTGFLLLVWN YKNTSSIPGP GYFLGIGPLI SYLRFLWMGI 70
#2  .......... .......... .......... .......... .......... .......... ..........
#3  .........Y ..I....... .......... .......L.. .E......S. .......... .HF.......
#4  .........Y ..I....... .......... .......L.. .E......S. .......... .HF.......
#5  .........* *NIS.....A .L.G...I.. .I.L..W... .ED...M... .......... .HF.......

                                                  4
#1  GSACNYYNKT YGEFIRVWIG GEETLIISKS SSVFHVMKHS HYTSRFGSKP GLQFIGMHEK GIIFNNNPVL 140
#2  .......... .......... .......... .......... .......... .......... ..........
#3  ........EM ....M..... .......... .......... .......... ..EC...Y.. ......D.A.
#4  .........M ....M..... .......... .......... .......... ..EC....D. ......D.A.
#5  .........M ....M..... .......... ..I..I...N ...C.....L ..EC...... ..M.....A.

          5
#1  WKAVRTYFMK ALSGPGLVRM VTVCADSITK HLDKLEEVRN DLGYVDVLTL MRRIMLDTSN NLFLGIPLDE 210
#2  .......... .......... .......... .......... .......... .......... ..........
#3  .......... .......... .......... .......... .......... .......... ..........
#4  .......... .......... .......... .......... .......... .......... ..........
#5  .....PF.T. .......... .......... .......... .......... .......... ..........

    6                                                   7
#1  KAIVCKIQGY FDAWQALLLK PDIFFKIPWL YRKYEKSVKD LKEDMEILIE KKRRRIFTAE KLEDCMDFAT 280
#2  .......... .......... .......... .......... .......... ...H...... ..........
#3  .......... .......... .EF...FS.. .K.HKE.... ...N...... ...CS.I... ..........
#4  .......... .......... .EF...FS.. .K.HKE...A ..DA...... ...CS.I... ..........
#5  S.L.H.V... .......... .......S.. .......... ..DA...... E..H..S... ....S...T.

          8                                                                   9
#1  ELILAEKRGE LTKENVNQCI LEMLIAAPDT MSVTVFFMLF LIAKHPQVEE ELMKEIQTVV GERDIRNDDM 350
#2  .......... .......... .......... .......... .......... .......... ..........
#3  .......... .......... .......... L......... .......... AIV......I ..........
#4  .......... .......... .......... L......... .......... GIV......I ..........
#5  Q..F...... .........V ...M...... ..I....... ...N...... ......Y... ..........

#1  QKLEVVENFI YESMRYQPVV DLVMRKALED DVIDGYPVKK GTNIILNIGR MHRLEFFPKP NEFTLENFAK 420
#2  .......... .......... .......... .......... .......... .......... ..........
#3  ...K...... .......... .......... .......... .......... .......... ..........
#4  ...K...... .......... .......... .......... .......... .......... ..........
#5  ...K...... .......... .F........ .......... .......... .......... ..........

    10
#1  NVPYRYFQPF GPGPRACAGK YIAMVMMKVT LVILLRRFQV QTPQDRCVEK MQKKNDLSLH PDETSGLLEM 490
#2  .......... .......... .......... .......... .......... .......... ..........
#3  .......... .......... .......... .......... .......... .......... ..........
#4  .......... .......... .......... .......... .......... .......... ..........
#5  .......... .......... .........I ..T....... ..Q.GQ.... .......... .H........

#1  IFIPRNSDKC FTK 503
#2  .......... ... 354
#3  .........S LDH 503
#4  .........S LEH 503
#5  .......... LEH 501
```

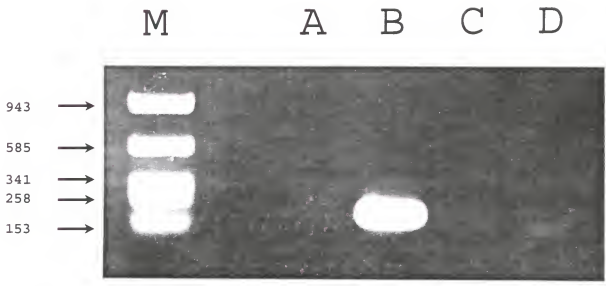


Figure 5- 4. RT-PCR analysis of P450 aromatase mRNAs in Day 60 pregnant pig placentae.

PCR amplifications were conducted on Day 60 placental RNA transcripts (pool of n= three pigs) using four different primer pairs (A lane, P1 & P2, expected size = 237 bp; B lane, P3 & P4, expected size = 193 bp; C lane, P4 & exon 1A- specific primer*, expected size = 259 bp; P4 and & exon 1B-specific primer*, expected size = 454 bp) at 30 cycles. (* see Figure 4-6).

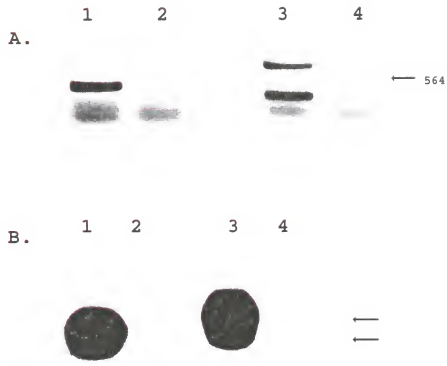


Figure 5-5. Detection of aromatase mRNA transcripts deleted in exons 4 to 6 in Day 12 pig embryos.

A. RT-PCR was performed with four primer pairs and 35 cycles of amplification. Lane 1, P5 - P6 (exon 5-8 of 33F clone, expected product = 411 bp); lane 2, P5 - P7 (exon 5-8 of P2 clone, expected product = 411 bp); lane 3, P8 - P6 (exons 3 - 8 of 33F clone, expected products = 653 bp, 296 bp for deleted clone); lane 4, P8 - P7 (exons 3 - 8 in P2 clone, expected size = 743 bp).

B. Amplification of exons 3, 7, and 8 sequences was confirmed by Southern hybridization with a [^{32}P] radiolabeled porcine P450 aromatase cDNA probe containing exons 5 to 7 of the 33F clone.

Figure 5-6. Comparisons of the 5' untranslated cDNA sequences for porcine and equine cytochrome P450 aromatase.

A. Two 5' untranslated cDNA sequences for porcine aromatase mRNA in Day 12 embryos (E1A, Top, see Figure 4-6A) and term placenta (Bottom, Hinshelwood et al., 1995) were aligned by use of the BESTFIT command of the GCG program.

B. Two 5' untranslated sequences for equine aromatase mRNA from Day 14 embryos (Top, see Figure 4-8B) and term placenta (Bottom, Hinshelwood et al., 1995) are compared. Primer sequences (P9 and P10) used for RT-PCR analyses (Figure 5-7) are indicated by bolded, underlined letters. The deletion is highlighted by shadowed dots (⁘). The presumptive splice junction for exons 1 and 2 are shown by arrows (▾) in A & B.

A.

```
 44 TGCGAAAGATCTAAAAACTAGCAGAAAGGATTTTCTACAGAGAAAGATAT 93
    ||*|*||||*||*||*||**|||||*|||||*|||||||||**||||||||
  1 TGAGGAAGACCTGAAGACGGGCAGAGAGGATCTTCTACAGCTAAAGATAT 50
                              ▾
 94 AAAGAAGGGTCACAACAAGACAGGACTTTAAATTGCTTCCTCTGAGATCA 143
    ||||||||**||||||||*||||*||||||||||||||||*|||||||||
 51 AAAGAAGGACCACAACAA.ACAGAACTTTAAATTGCTTCCCCTGAGATCA 99

144 AGCAATGCAAGATG 157
    |||||**|||||||
100 AGCAACACAAGATG 113
```

B.

```
                                P9
 44 CACAACTGAACTGGTCACAGTAACAGCTGCCACCAGTGTCTCAGCTGTCT 93
    ||||||||||||||||||||**||||||||||||||**||||||||||||
  1 TACAACTGAACTGGTCACAGGTACAGCTGCCACCAGT..CTCAGCTGTCT 48

 94 CCTGCCTCTCCAAGATAAGCTCCAAGCTTAATAAGGTCAAAAACTCAGAC 143
    |||||||||||||||||||||||||||||||||||
 49 CCTGCCTCTCCAAGATAAGCTCCAAGCTTAATAA.................. 82
                                P10      ▾
144 GTCTTCAGGATCCAAGCAGGTGAGGAGGACGGCTTTGGCAGGCCTTTACA 193
                                                |||||||||
 83 ..........................................GCCTTTACA 91

194 TTGCTTCGCCTGAGATCAAGGAGCACAAGATG 225
    |||||||||||||||||||||||||||||||
 92 TTGCTTCGCCTGAGATCAAGGAGCACAAGATG 123
```

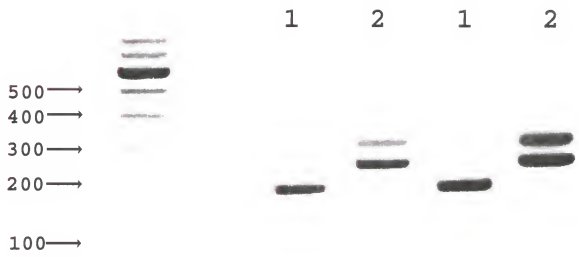


Figure 5-7. Detection of two cytochrome P450 aromatase mRNA variants in periimplantation equine embryos.

RT-PCR was carried out with a primer designed from the exon 2 sequence of equine aromatase (see Figure 4-9; 5' CTGGTATTGAGGATGTTTCTTCATG 3') and a second primer (lane 1, P10, expected product size, 198 bp; lane 2, P9, expected product size, 309 bp), and RNA isolated from d14 equine embryos (n=2). Two identical PCR reactions were conducted for each primer pair. The numbers on the left side indicate the sizes (bp) of the DNA standards.

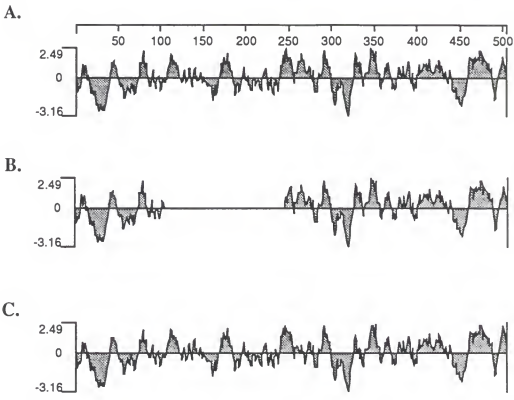
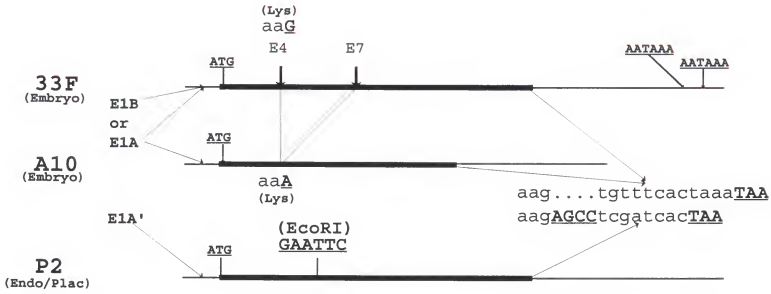


Figure 5-8. Hydropathy analysis of three porcine cytochrome P450 aromatase proteins.

The comparison was calculated by the algorithm of Kyte and Doolittle. A. Protein encoded by 33F clone, B. Protein encoded by A10 clone, C. Protein encoded by P2 clone, respectively.

Figure 5-9. Structure of cDNAs encoding three isoforms of porcine cytochrome P450 aromatase.

The area encoding the P450 aromatase protein is shown as a thick black line with translation initiation (**ATG**) codons indicated. 5' untranslated aromatase exons (E1A, E1B, and E1A') and the first and second polyadenylation signal sequences (**AATAAA**) used for the 33F clone are shown, respectively. The frame shift of downstream codons resulting from an insertion of four nucleotides (**AGCC**) in the P2 clone is indicated. The last two nucleotides (aa) at the end of exon 3 is spliced to the first nucleotides of exon 4 (**G**) in the 33F clone and exon 7 (**A**) in the A10 clone, respectively, to form the same codon specificity for lysine (Lys), respectively. The additional EcoRI site (**GAATTC**) in exon 6 of the P2 clone is also shown.


(Lys)
aaG
E4
E7
ATG
AATAAA
AATAAA
33F
(Embryo)
E1B
or
E1A
ATG
A10
(Embryo)
aaA
(Lys)
aag....tgtttcactaaaTAA
aagAGCCtcgatcacTAA
E1A'
(EcoRI)
GAATTC
ATG
P2
(Endo/Plac)

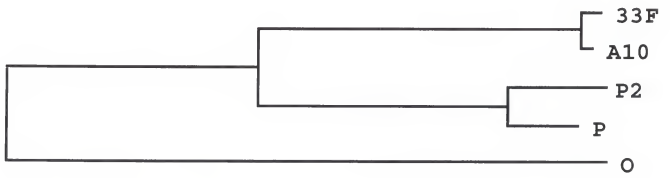


Figure 5-10. Evolutionary tree for porcine cytochrome P450 aromatases.

This tree was constructed by use of the neighbor-joining method (Saitou and Nei, 1987) and was based upon the five-known polypeptide sequences of porcine aromatase. The distance of the line indicates the differences of amino acid sequences among the isoforms. P and O represent aromatase protein sequences from term placenta and ovary (Corbin et al., 1995), respectively.

Discussion

In the present chapter, I describe the isolation of two additional types of porcine cytochrome P450 aromatase cDNAs from Day 12 blastocysts (A10 clone type) and Day 60 pregnant pig endometrium and placenta (P2 clone type). The A10 and P2 clones encode aromatase proteins of 354 and 503 amino acids, respectively. The collective results from restriction-endonuclease digestion and DNA sequencing of forty-four cDNA clones and from RT-PCR analysis identified an interesting developmental switch in expression of the two different encoded aromatase isoforms. The transcript exemplified by the 33F clone is highly expressed in periimplantation porcine blastocysts and is the major aromatase mRNA expressed in endometrium and placenta during early pregnancy; in contrast, the P2 transcript is the major aromatase mRNA in endometrium and placenta at midpregnancy. These results confirm that the lower or lack of mRNA amplification in Day 60 placenta and endometrium, in the experiments of Chapter 4, using the E1A -E3 and E2-E3 primers was not due to less efficiency of PCR amplification, but rather, to no or very rare expression of the 33F mRNA.

Nucleotide sequencing revealed that the A10 clone was deleted in exons 4-6 but was otherwise identical to the 33F clone, except for one nucleotide substitution in the exon 7 region. We speculate that deletion of exons 4-6 resulted from an alternative exon splicing mechanism, which has not previously been reported for aromatase or other steroidogenic enzyme genes. Although the A10 clone was the only one found to be deleted in exons 4-6 among ten PCR clones isolated from blastocysts, the results of RT-PCR confirmed

significant expression of this mRNA type in the blastocyst. Hydropathy analysis indicated that the amino acids encoded by the deleted region in the A10 clone are neutral (Figure 5-8). This suggests that deletion of this region may not significantly affect protein structure or perhaps enzyme activity. It is of interest to note that there are numerous unknown phenolic compounds other than estrogens, which are produced by periimplantation porcine blastocysts via the actions of as yet unidentified enzymes (Gadsby et al., 1980; Fischer et al., 1985). The activities of catechol estrogen synthetase (estrogen 2/4-hydroxylase) (Mondschein et al., 1985) and estrogen 15α-hydroxylase (Chakraborty et al., 1990) in periimplantation pig blastocysts are highly correlated with the transient expression of aromatase and 17α-hydroxylase enzyme mRNAs and proteins (Conley et al., 1992; Ko et al., 1994; Conley et al., 1994; Green et al., 1995). Osawa et al. (1993) demonstrated that aromatase is responsible for the synthesis of catechol estrogen by the human placenta. Therefore, it is interesting to speculate that the 33F or A10 mRNAs which are highly expressed in blastocysts as compared to the P2 clone type, may encode for enzymes that are involved in the synthesis of one or more of the compounds described above.

The P2 mRNA is the major aromatase transcript present in endometrium and placenta during midpregnancy. The deduced amino acid sequence of the protein encoded by the P2 clone has 92.6 % and 98.4 % identity compared with that for the 33F cDNA and the term placenta cDNA, respectively (see Table 5-2). Only seven amino acid differences were noted between the two placental aromatase proteins. The results from complete DNA sequencing of six cDNA clones from endometrium and placenta demonstrated near total consistency of their nucleotide sequences. However, it is not clear whether the aromatase mRNAs in

mid-pregnancy vs. term placenta are the same or different, since the comparable nucleotide sequence of the term placental clone has not been published or released by Genbank. Neither the E1A or E1B aromatase exons in periimplantation stage blastocysts are likely to be used for placental aromatase transcripts at midpregnancy, based upon the RT-PCR results from the previous Chapter 4 and these presented here. The putative structures of the three isoforms of porcine aromatase transcripts are summarized in Figure 5-9.

The deduced amino acid sequence from aromatase mRNA expressed in pig ovary was recently reported (Corbin et al., 1995). Expression of the ovarian cDNA clone in COS cells identified a lower specific activity for this protein than for the term placental aromatase isoform (Corbin et al., 1995). Interestingly, the one aromatase cDNA clone so far isolated from peri-estrus pig ovary appears to be of the type represented by the 33F clone based upon DNA sequencing of exons 2-3 regions and EcoRI enzyme digestion. This suggests that the 33F clone-encoded aromatase may also be responsible, in part, for ovarian synthesis of estrogens. It is likely that different types of aromatase transcripts may be amplified to differing degrees depending on the primers used for RT-PCR. Therefore, it is not clear at present, which are the major aromatase transcripts in ovary tissue.

A total of five different isoforms of porcine aromatase encoded by cDNA clones have now been reported here and by others (Corbin et al., 1995). Comparisons of the amino acid sequences of these isoforms indicated greater than 90 %, similarity with the exception of the ovarian aromatase protein. As shown in Figure 5-8, hydropathy analysis of the three aromatase proteins identified here also demonstrated significant degree of similarity among all three. An evolutionary tree was constructed based upon the polypeptide sequences of

the five proteins and this indicated that the blastocyst and placental aromatase protein were closely related to each other and more distantly related to the ovarian protein (Figure 5-10). Analysis of protein sequence suggests that the exon 5 sequence is identical among cDNA types, exons 9-10 regions, encoding the heme-binding domain are highly conserved, and that the other exon regions are more divergent. Interestingly, a putative peptide sequence for phosphorylation by cAMP-dependent protein kinase, highly conserved for aromatase from several species (Figure 4-3), is present in the proteins encoded by all five cDNA types. The activity of human cytochrome P450 17α-hydroxylase is known to be increased after phosphorylation by a cAMP-dependent protein kinase (Zhang et al., 1995). This suggests, but does not prove, a similar role for phosphorylation in aromatase function.

Only a single isoform of cytochrome P450 aromatase has been reported for each of five mammalian, two avian, and two piscine species, which contrasts with the five different isoforms of porcine aromatase described here. Multiple isoforms of 3β-hydroxysteroid dehydrogenase which are expressed in a tissue-specific manner are encoded by multiple genes in the rat, mouse, and human (Payne et al., 1995). We have obtained additional evidence that the 33F clone isolated from pig blastocysts and the P2 clone isolated from mid-pregnancy placentae are encoded by different genes which are highly conserved in structure and DNA sequence (Chapter 6). It is therefore interesting to speculate that multiple tissue-specific aromatase isoforms in other species that are as yet unidentified, may exist. Results from RT-PCR assays indicate that there are at least two equine aromatase transcripts with different 5' end untranslated regions in Day 14 equine blastocysts. Whether the two 5' ends identify different aromatase isoform-encoding transcripts is unclear. The 5' ends of

porcine embryo and placental mRNAs (Figure 5-6A) encoded by two related genes are similar though no identical in their DNA sequences. Therefore, it is possible that there are at least two equine aromatase genes, expressed in embryos and placenta, since the known multiple 5' ends of aromatase cDNAs alternatively spliced in the human, and bovine, and porcine blastocyst do not have any similarity with each other. At present, the molecular mechanisms underlying developmental expression of multiple isoforms of aromatase and the physiological function(s) of each isoform is unclear.

In conclusion, the results presented in this chapter provide additional information regarding a) the transient expression of the porcine aromatase gene that is responsible for the synthesis of estrogens and perhaps other steroidal molecules by periimplantation pig blastocysts; and 2) the molecular mechanisms underlying the tissue-specific and developmental expression of this gene in other species.

CHAPTER 6
MOLECULAR CLONING AND STRUCTURAL CHARACTERIZATION OF PORCINE CYTOCHROME P450 AROMATASE CHROMOSOMAL GENES: EVIDENCE FOR THE EXISTENCE OF MULTIPLE, CLOSELY RELATED GENES THAT ENCODE DEVELOPMENTAL AND TISSUE-SPECIFIC ISOFORMS OF AROMATASE

Introduction

A single enzyme complex of cytochrome P450 aromatase, derived from the single copy CYP 19 gene, has been considered to be solely responsible for the synthesis of estrogens from androgen substrates such as androstenedione, testosterone, and 16α-hydroxyandrostenedione (Corbin et al., 1988; Simpson et al., 1994). Previous identification of only a single isoform of aromatase for each of six mammalian, two avian, and two piscine species and an evidently single copy aromatase gene from several species (Means et al., 1989; Harada et al., 1990; Toda et al., 1990; Terashima et al., 1991; Tanaka et al., 1995) provided support for the existence of a single form of aromatase protein and chromosomal gene. However, tissue-specific and developmental expression of multiple isoforms of other steroidogenic enzymes with different activities for substrates has been demonstrated, although the physiological significance of these isozymes is not yet clear (White, 1994). Isoforms of steroidogenic enzymes, where identified, were typically found to be derived from multiple genes. The genes for the isozymes of steroid 11β-hydroxylase ($P\text{-}450_{11\beta}$, Mornet et al., 1989) and 3β-hydroxysteroid dehydrogenase (3β-HSD, Labrie et al., 1994; Payne et

al., 1995) are closely linked on chromosomes and have high sequence similarity with each other, whereas the genes for the 17β-hydroxysteroid dehydrogenase (17β-HSD) isozymes are neither physically linked nor exhibit significant homology with each other (Luu-The et al., 1990; Wu et al., 1993; Casey et al., 1994). Pseudogenes for the steroid 21-hydroxylase (Higashi et al., 1986) and17β-HSD (Luu-The et al., 1990) gene families were also found.

The nucleotide sequences of aromatase coding exons (2-10) are highly conserved for several species (see Chapter 4), whereas the 5' untranslated exons which are generated via alternative promoter usage and differential splicing mechanisms are divergent between tissues and mammalian species (Harada et al., 1992; Mahendroo et al., 1993; Harada et al., 1993; Toda et al., 1994; Honda et al., 1994; Hinshelwood et al., 1995; Chapter 4). It seems likely that alternative usage of 5' untranslated exons in aromatase transcripts is common for several of the domestic species as well as the human. Although recent evidence suggests the existence of an aromatase pseudogene in the bovine genome (Furbaβ and Vanselow, 1995), there have been no reports demonstrating the presence of multiple functional genes for P450 aromatase for any species. However, tissue-specific and developmental expression of multiple isoforms of porcine aromatase has been indicated in the previous two Chapters (Chapters 5 and 6) and by others (Corbin et al., 1995).

Results from previous studies demonstrated a developmental switch in the expression of mRNAs encoding two isoforms of porcine aromatase in endometrium and placenta (Chapter 5), as well as the transiently high expression of an embryo aromatase isoform in periimplantation pig blastocysts (Conley et al., 1992; Ko et al., 1994; Green et al., 1995; Chapter 4). Corbin et al. (1995) demonstrated that a term placental aromatase isoform,

which is homologous or perhaps identical to the midpregnancy placental aromatase isoform (P2 clone type) previously identified (Chapter 5), had much higher enzyme activity than a porcine ovarian aromatase isoform. An additional mRNA type, potentially encoding yet another embryonic isoform of aromatase, which was deleted in exons 4-6 but otherwise identical in nucleotide sequence to the embryo cDNA, was identified in periimplantation pig blastocysts. It seems likely that the internally deleted embryonic aromatase transcript results from an alternative RNA splicing mechanism(s), which has not been previously reported for any species. It is speculated that aromatase and aromatase-related proteins expressed in pig blastocysts may be involved in the synthesis of unknown phenolic compounds whose biological functions have yet to be investigated (Gadsby et al., 1980; Fischer et al., 1985) or catechol estrogens which have been shown to be synthesized by human placental aromatase (Osawa et al., 1993).

Therefore, the present experiments were carried out to clone and characterize the postulated multiple porcine aromatase chromosomal genes as a requisite step towards understanding the molecular mechanisms involved in the transient expression of aromatase in porcine blastocysts during the periimplantation period as well as the developmental switch in expression of two aromatase isoforms in endometrium and placenta. In this chapter, evidence is presented for the existence of multiple porcine aromatase genes, suggesting that: a) the expression of the aromatase isoforms encoded by diverse mRNA transcripts are derived from these multiple porcine cytochrome P450 aromatase genes and, b) a single embryo-type aromatase chromosomal gene which is transiently and highly expressed in pig blastocysts during the periimplantation period, exists in this species.

Materials and Methods

Molecular Cloning of Porcine Aromatase Genomic DNA Fragments

A porcine genomic DNA library made from adult pig liver (Clontech, Palo Alto, CA) was screened using a [^{32}P]-radiolabeled human aromatase cDNA fragment spanning exons 3-10 of the human gene (Chen et al., 1988) (for K and B clones) or a porcine aromatase cDNA fragment encoding exons 1A-5 of the 33F cDNA clone (A1, A4, C3 and S1 clones), prepared by nick translation. Hybridization screening of 3-5 X 10^5 phage clones on replica filters was conducted using standard procedures described in Chapter 4 (Materials and Methods). Hybridization was carried out in 6X SSC/5X Denhardt's solution/0.5X SDS/yeast RNA (250 μg/ml) containing ~0.5 $\times$ 10^6 cpm/ml probe for 18 hours at 62 °C. Filters were washed in 2X SSC/0.1% SDS for 30 minutes at room temperature and in 0.2X SSC/0.1% SDS for 1 hour at 62 °C before exposure to X-ray film.

Restriction Endonuclease Mapping

The locations of restriction endonuclease cutting sites (Sal I, Eco RI, and Hind III) in phage DNAs were determined by: a) single or double digestions followed by Southern blot analysis using [^{32}P]-radiolabeled human or porcine aromatase cDNA probes, b) comparisons, where overlapping, with detailed restriction maps for subcloned fragments of phage clones, and c) confirmation by computer analysis of DNA sequences that were subsequently obtained from subclones.

PCR

Polymerase chain reaction for exon 4 and exon-intron boundary sequences using phage clones as templates (1 minute at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C)

was performed in a 50 μl volume containing [50 mM KCl, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 0.001% gelatin], 50 nmol dNTPs, 50 pmol of each primer, 1 unit of Taq polymerase (Boehringer Mannheim), and 100 ng of purified phage DNA as template. PCR amplification of exons 7-9 using genomic DNA as template (30 seconds at 94°C, 40 seconds at 65°C, and 8 minutes at 68°C for ten cycles; and 30 seconds at 94°C, 40 seconds at 65°C, and 8 minutes and an additional 20 seconds in each subsequent cycle at 68°C for 15 cycles) was carried out in a 50 ul volume containing the buffer described above, 50 nmol dNTPs, 50 pmol of each primer, 3 units of a mixture of Taq DNA polymerase and Pwo DNA polymerase, and 1μg genomic DNA isolated from Day 30 pregnant endometrium (one pig) as template. PCR products were subcloned in the PCR II vector and transformed into competent $INV_{\alpha}F'$ cells (TA cloning kit, Invitrogen). The sequence of each oligonucleotide primer used [intron sequences immediately upstream and downstream of exon 4 (a and b primers) and exons 7-9 (c and d primers)] were as follows:

a: 5' GAGAAGACTATAGCCACATACTTAA 3'

b: 5' GTTTCTGACCCTGAGCAATTTATCC 3'

c: 5' GGATTTGAAAGAGGACATGGAAATTCTG 3'

d: 5' CTCAAGAGTAAATTCATTGGGCTTGGGG 3'

Southern Blot Analysis

Genomic DNA isolated from endometrium of a Day 12 pregnant pig was digested overnight with restriction endonucleases, electrophoresed in a 1.2 % agarose gel, and transferred to a Biotrans nylon membrane using the TurboBlotter (Schleicher and Schuell, Keene, NH). The membrane was hybridized as described in Chapter 4 using pig aromatase-

specific cDNA probes (Figure 6-10). After washing the membrane once in 2X SSC/0.1% SDS for 20 minutes at room temperature and once in 0.2X SSC/0.1 SDS for 20 min at 62 °C, the membrane was exposed to X-ray film for 2-3 weeks at -80°C using intensifying screens.

DNA sequencing

Double-stranded DNA sequencing was performed using the Sequenase-based DNA sequencing kit (United States Biochemicals, Cleveland, OH).

Results

Six phage clones containing porcine cytochrome P450 aromatase chromosomal DNA fragments were obtained after screening of a porcine genomic DNA library using [^{32}P]-radiolabeled cDNA probes encoding human (B and K clones) and porcine aromatase (A1, A4, C3, and S1 clones), as described in the Materials and Methods. Results obtained from restriction endonuclease mapping and DNA sequence analysis of the six clones indicated that these represented three different aromatase chromosomal genes (Figure 6-1). Three clones (A4, B, and K), designated as the type I porcine aromatase gene, collectively encompassed exons 4-10 of the embryo-expressed gene and the nucleotide sequence of each exon was found to be identical to that of the 33F cDNA clone previously isolated from Day 12 embryos. The S1 clone (designated as the type III porcine aromatase gene) contained two exons (exons 2 and 3, by analogy to the human gene) which encoded a peptide sequence nearly identical to that of pig ovarian aromatase (Corbin et al., 1995). The amino acid sequence encoded by an exon in the C3 clone (designated type IV porcine aromatase) was identical to that of ovarian aromatase, but the nucleotide sequence of a downstream exon was identical to that of embryonic aromatase (33F clone type) cDNA. The A1 clone likely

belongs to the type I aromatase locus based upon the high degree of similarity of its restriction enzyme map with those of the A4, B, and K clones.

DNA sequencing of all exons and exon-intron boundaries revealed that the size of each exon, except for exon 2 in the S1 clone, is identical to that for the corresponding human aromatase gene exon (Harada et al., 1990) and conserved nucleotide sequences for RNA splicing were found for all exons (Figure 6-2). The two polyadenylation signals identified in the embryo 33F and 34B cDNA clones (Chapter 4) were found in the exon 10 region of the type I aromatase gene (Figure 6-2). The size of each intron for the type I porcine aromatase gene except for intron 3, was determined by restriction enzyme mapping and/or PCR (Table 6-1). The I.1A and I.1B exon sequences alternatively utilized in the embryonic aromatase transcript (Chapter 4) were localized at 4.3 and 4.1 kb upstream of the exon I.2, respectively, by the PCR method. Therefore, the overall size of the type I porcine aromatase gene was determined to be a minimum of 28.4 kb.

The exon sequences obtained from the phage genomic DNA clones were compared with cDNA sequences encoding two aromatase isoforms identified in Chapters 4 and 5 (Figure 6-3). Similarly, the deduced protein sequences encoded by genomic exons were compared with those of five aromatase isoforms previously identified in Chapters 4 and 5 and by others (Corbin et al., 1995) (Figure 6-4). A deletion of six nucleotides (#1' in Figure 6-3), which causes a two amino acid deletion (Figure 6-4, #1') and which is characteristic of the ovarian aromatase protein isoform (#6 in Figure 6-4), is apparent in the S1 clone. The amino acids encoded by the putative exons 2-3 of the type III aromatase gene (S1 clone) are identical to ovarian aromatase except for a single amino acid difference at the 3' end of exon

2. The nucleotide sequences of exons 4 through 10 of the type I aromatase gene are identical to that of the 33F clone. A single nucleotide difference in exon 7, probably due to PCR error or DNA polymorphism, was noted between the type I genomic sequence and the A10 cDNA clone isolated from embryos. A four nucleotide insertion in the P2 clone characteristic of the aromatase transcripts expressed in midpregnancy and/or late pregnancy was not present in the two embryo aromatase cDNAs and the type I genomic sequences (Figure 6-3).

Four phage clones (A1, A4, B, and K) appear to be representative of a single gene locus encoding the embryonic aromatase isoform. This is based upon the high degree of similarity of their restriction enzyme maps and their exon nucleotide sequences. Two clones (S1 and C3) appear to be derived from distinct genes but show similarity in their restriction enzyme maps, where overlapping, and are identical in the exon 3 nucleotide sequence shared by both clones. The restriction maps of the region encompassing exon 4 of the A4, K, and C3 clones are quite similar to each other. Thus, to further examine whether the six phage clones originate from a single gene or multiple genes, PCR amplification of the exon 4 region was performed using the individual phage clones as templates and including the S1 clone as a negative control. Primers were designed from the intron sequences immediately upstream and downstream of exon 4 as obtained by sequencing a plasmid subclone derived from the A4 phage clone. Surprisingly, PCR products were obtained from all of the phage clones that were shown to contain exon 4, in contrast to the S1 clone as control (Figure 6-5). In addition, the DNA sequences of the exon 4 PCR products subcloned from all three phage clones (n=2 subclones for each phage template analyzed) were identical.

Southern blot analyses of the six phage clones was carried out using a radiolabeled

probe containing exon 4 and its boundary sequences (Figure 6-6) and extending upstream into the adjacent intron (Figure 6-7). The expected size (~1.0 kb) of an EcoRI fragment from the A4 and K clones, consistent with the restriction maps for the two phage clones (Figure 6-1), that hybridized with the probe was observed; whereas, a smaller hybridized fragment (~ 0.8 kb) was detected for the C3 clone. Two PstI fragments were detected by hybridization to the A4 and K clones due to the presence of internal PstI site in the third EcoRI fragment of the A4 clone, which was used as a probe, but only a single hybridization fragment was detected for the C3 phage clone. Whereas the C3 clone exhibited slight differences in DNA sequence (as manifested by RFLPs) from the A4 and K clones surrounding the exon 4 region, the general hybridization patterns for the EcoRI and PstI fragments of all three clones were very similar (Figure 6-7). Southern blot analysis confirmed the absence of exon 4 sequences in the S1, A1, and B clones and ruled out the possible presence of additional, alternatively spliced exons 4 in all of the phages. An additional Southern blot analysis was conducted for the six phage and two plasmid subclones to demonstrate the relative lack of additional alternatively utilized exons using a probe spanning exons 5-7 of the embryonic aromatase cDNA clone (Figure 6-8). The results demonstrated that the hybridization patterns to restriction enzyme-digested fragments agreed well with the restriction enzyme maps shown in Figure 6-1, and again, eliminated the possibility of the presence of additional alternatively utilized exons in these genomic clones. Genomic Southern Blot analysis also was performed on pig chromosomal DNA using three different probes representing different regions of the embryonic porcine aromatase cDNA/gene (Figure 6-9). Three hybridized bands of expected sizes were detected in the EcoRI digested DNA but no hybridized band

of the expected size (~4.5 kb) was detected in the HindIII digested DNA using an exon 5-7 probe, and one or two additional bands were detected after hybridization using each of the three probes.

To confirm the presence of additional pig gene(s) encoding aromatase, PCR amplification of chromosomal DNA was conducted using a pair of primers designed from regions where nucleotide sequences were highly conserved between the embryonic and placental aromatase cDNA clones. Two major types of PCR product that differed by about two hundred base pairs in length were co-amplified by this procedure and these were subcloned into a plasmid vector. Results from DNA sequencing of eight such subclones revealed that all four subclones of the larger PCR product contained an identical DNA sequence to that of embryonic or type I aromatase. All four subclones of the smaller PCR product exhibited the placental aromatase (P2 clone type) cDNA sequence (Figure 6-10). Surprisingly, even the sequences of the introns were highly homologous between these two genes.

The sequence of the region upstream of exon 2 of the ovarian aromatase gene (type III) which by analogy to the human should represent the ovarian promoter, was obtained (Figure 6-11). A putative TATA box sequence was found at forty-four base pairs upstream of the presumptive exon-intron splice junction utilized for embryo, endometrium and placental aromatase transcripts. The DNA sequences for a consensus CRE (cAMP response element) and SF-1 (Steroidogenic factor-1) binding site, which have been found in all known 5' sequences of the ovarian aromatase promoter, were identified (Figure 6-11).

Figure 6-1. Restriction endonuclease cleavage maps of pig genomic DNA clones encoding multiple isoforms of cytochrome P450 aromatase.

Six phage clones containing genomic exons encoding porcine cytochrome P450 aromatase were analyzed for locations of restriction enzyme cleavage sites and exons. The designation of each exon (by analogy to the human aromatase gene) is indicated on the top of the thick vertical bar. The positions of sites for Sal I and Hind III (top), and EcoRI (bottom) are marked by thin vertical lines. Sal I cleavage sites are indicated by an S. The regions subcloned and used as probes for Southern blot analysis are indicated by numbers with underline. Four clones (A1, A4, B, and K) span exons 4 to 10 of the embryo type I porcine cytochrome P450 aromatase gene and the nucleotide sequence of each exon is identical to that of the 33F cDNA clone isolated from Day 12 embryos. The S1 clone (type III porcine cytochrome P450 aromatase gene) contain exons 2 and 3 encoding polypeptide sequences, except for one amino acid substitution in exon 2, to that reported for pig ovarian aromatase (Corbin et al. 1995). The C3 clone (type IV porcine cytochrome P450 aromatase gene) contains coding exons 3 and 4, and the restriction enzyme map of the region where overlapping is very similar to that of S1 clone. The exon 3 peptide sequence of the C3 clone is identical to that of aromatase from ovary (Corbin et al. 1995); whereas, the exon 4 sequence is identical to the corresponding sequence of the embryo 33F clone. Exons marked with single stars (*) were subcloned into plasmid vectors and sequenced; exons marked with double stars (**) were subcloned after PCR amplification and sequenced; and exon 4*** was both subcloned and PCR amplified prior to its sequencing.

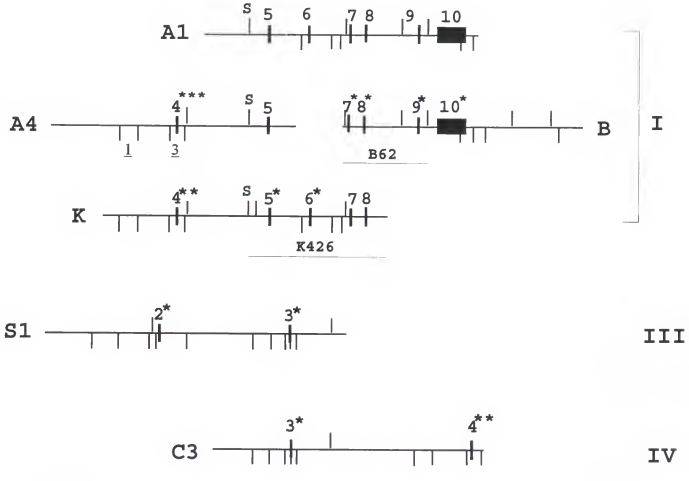

A1
S
5
6
7 8
9
10
A4
4***
S
5
7*8*
9*
10*
B
I
1
3
B62
K
4**
S
5*
6*
7 8
K426
S1
2*
3*
III
C3
3*
4**
IV
2 kb

Figure 6-2. DNA sequences of exons and exon-intron boundaries for chromosomal gene segments encoding different isoforms of pig cytochrome P450 aromatase.

Nucleotide sequences of exons 4 to 10 of the type I porcine cytochrome P450 aromatase gene and exons 2 & 3 of the type III aromatase gene are shown. The first and last six nucleotides of each exon are highlighted as bolded, underlined letters. The polyadenylation signals for the type I aromatase gene and the translational initiation codon for the type III aromatase gene are indicated by double underlines.

Exon I.4

AAATGTG TGTTTGTGTG
TATGTGTTTG TGTGTGTGTG TGTTGTGTGC GTAGTGTGTG TGTTTTAGCT
TAAGTGATGC TCACACAGAG AGCTAGTAGC TAAAAATGTT TCAACTTGAG
GAGGTTCTTG TGCTCCTGAA ATAACACAAG TGCCTATTTA ATCTGAACAC
TAGAAATTAT GCACTATGGT CAGATCAATA TGCAGTTGCC ACCAGGTAGT
CAAACTCACT AAACTGAGTT TCTGACCCTG AGCAATTTAT CCCAAGGATT
TCTTTTT**CTG CAG**GTCCTCA AGTGTGTTCC ATGTAATGAA GCATAGTCAC
TACACATCCC GATTTGGCAG CAAACCTGGG TTGCAGTTCA TTGGCATGCA
TGAGAAAGGC ATTATATTCA ACAATAATCC AGTCCTCTGG AAAGCTGTTA
GAACTTATTT TATGAAAG**GT ATTT**AAGTAT GTGGCTATAG TCTTCTCTTT
TGTTTATGTC TATTCCTGTT ATGAATT

Exon I.5

GTTTTATT
TTAAAAATCC ATTCATTGAT TCATTTTAAA TCATAAATAG GAGACATTCT
AGAAGGAAAT TAGAAGAGTG TAGAAAAGGT AGAAAGAAGA ATGTGAAACT
TTCCACCCAC TTATCAAACG AAAATGTGTG GCCAATTAAG CTCCAAACAG
AAAGAGCCAA AGTCCTCTGA TGGTGCTTGT GTGCCCTGGA GCTGGAGGAA
GCTCACTCTC TCTGATTGAT GTGCACC**CTT CAG**CTCTGTC CGGCCCTGGC
CTGGTGCGCA TGGTGACCGT CTGTGCCGAT TCCATCACCA AGCACCTGGA
CAAGCTGGAG GAGGTCCGCA ATGACTTGGG CTACGTGGAC GTGTTGACCC
TCATGCGGCG CATCATGCTG GACACCTCTA ACAACCTCTT CCTGGGGATC
CCGTTGGATG **GTATGG**AAAC TTTCATCTCC TGACTTGACG TTCTGCCCTT
CCTTGAGCAG GACACAAGAA GGGTAATGGA CTCAAGTAAA GATAGGCTCT
TTTGCACACT GCTCTTCTTT AAACATTTCT AAGGAGCCCT AAAATGTCTT
CCAGGTAGGT CTGCCTTGTT CACGAACAGG ATCTTGTATA TCATTGCAGG
GGCCCTCTTC TATTTCTTGT GTTTCTCCTT A

Exon I.6

ATTAAAT TAGTGTTGGT
TTGTTTAATG GCATTTACAT GGCTGAAACA CCTACTGATG GTGGAACCTT
GCCCTGCTTA GAATCTCAGA TGGTAGGAAA CTCTATTGAG GACCTTCTCT
GATCAGTGTA TCTTTCTTGG GCTTCCATTT TTGCTCAGCT GCTCTGCTCT
GTTTCTGTTT **TTCCAG**AAAA GGCCATTGTG TGTAAAATCC AGGGTTATTT
TGATGCATGG CAAGCTCTCC TTCTCAAACC AGACATCTTC TTTAAGATTC
CTTGGCTGTA CAGAAAGTAT GAAAAGTCTG T**GTAAGT**AAC ACATTTTAGA
AAATATATCG GTGAGACTGG ATTTATTTGT ATTGTCTTAG ATATTGTTGG
CCTCTTCAAC AAAGTTTCTC CTCTCTAGAA TCATAAGGGA GCTGTTAAGT
TGCTGATGAA ACACTTCTTA CAGCAATTCT GGTGTGCATA GCCAGTTCAA
AGAGTAAAGA AAGCCTTTTG CCTGTGCAGT TGTCTAGCCA GAGGGCTGAC
TGGGTGGTGT GAGAGCTCCA TGTCTGGAAA TTGGACACTT CAGAGTATTT
TAAGATGTAG AGAAGGGTTA GATGAACCTA TGATTATTAT TTATCAAAAA
TTCTTTGCAC GTCTGAAAGA A

Exon I.7

TG CTGGCCCAGT
CAAATTTCAG AAAACCTGAT CAGATTTAGC CTTTTGGTAC CAGTTTTTAA
TCCCTAACTT GAGGTTATAG CCTTGATTTT ACTACTAGTA CCTAGACATC
TTAGCTAACT CTGTATAACT GTATATACAT ATATATATAA TTTAAGGATA
TTTGTGAAGA ATTAAGTATA AATTCAGTTA TGAGCACTAA TAGCATCAAC

Figure 6-2 -- continued.

TAAAGCAGAT CATTTATGTC AACAATTGGC AGAGTAATCC ATTTATCTGT
GTACAGATAG ATTATATTTT CACTGCCTTT **TGCAG**AAAGG ATTTGAAAGA
GGACATGGAA ATTCTGATAG AAAAAAAAAG ACGCAGGATT TTCACAGCAG
AAAAACTGGA AGACTGCATG GATTTCGCCA CTGAGTTGAT TTTGGCTGAG
GTATGGATCT GGACTATCCA TAATTCCCAC ACAACACAGT GTATGATTGT
TTACAGGTGT CTAAAAATTC ACTCCTGTTA TCTGTTGCAT GTTTCTGCTT
AATAAAGCAT CTGCTCTAGT A

Exon I.8

ACTGCTGTGG TTCCAGCAGC TGCTGCGACT
CCCTGACTGC TGTGGCACAG GTTCAACCCC TGGCACAGGA ACTTCCACAT
GTTGTAGCAT GGCCCACTAA AAAAAATTTT TTCAGCTTTG AGCTGAAGAT
TGTTACAAAA AAGTCCATTG ACAATCCCAA GGATACATAA TCTCTACAAT
TTCATTTTGC TGACCTTGTT GATCCTTGAG TGACACCTTC CCCCTTGTTC
TGAAACATTC ATTCACTTTT CCAATGTTTC CCCGCCTCCT TTCCATT**GCT**
CAGAAACGTG GTGAACTGAC AAAAGAGAAT GTGAACCAGT GCATACTGGA
AATGCTAATT GCAGCACCAG ACACCATGTC TGTCACTGTG TTCTTCATGC
TGTTTCTCAT TGCAAAGCAC CCCCAGGTTG AAGAGGAACT AATGAAGGAA
ATCCAGACTG TTGTTG**GTAA GA**ATGTATCA AACAAATAAT AGAGTTTAGA
AAACAACTCC TTCTGAATAT CAGCTTCAGT GTCCTAAGAA TCCTATGTCA
AAATCTTCAT TACAATCCAC CTAAAGAACA ATAAAACAAG TCACTTGGTC
ACAATGTCAT TAGGCCAGAC AGGTTAAAAA GGTAGTACTT CCCGGGTTTT
TCTAAGATAA ACAGTATATG TTTATTGGAA AAATTCAAAG AATAAAGAGA
TGTGTAAAGC AGAAAGTCAT ACCCCTGATC CATCACACTC TCTACCCCAG
CCTCATCCCA TTCCCAGGGG GAATCACCAT TAATA

Exon I.9

CAAAC CAAGACTGAA CTAACCTTTA
ACTGTACTGA TCTGAAACTA GGACTTTTAT GCCCATCACA TATAGAAAAT
AACATGCCCT CCTTTTCATC TGCTC**TGACA G**GTGAAAGAG ACATAAGGAA
TGATGACATG CAAAAACTCG AAGTGGTGGA AAACTTTATT TATGAGAGCA
TGAGGTACCA GCCTGTCGTG GACCTCGTCA TGCGAAAAGC CTTAGAGGAT
GATGTCATCG ATGGCTACCC GGTGAAAAAG GGAACCAACA TTATCCTGAA
TATTGGAAGA ATGCATAGAC TCGAGTTTTT CCCCAAGCCC AATGAATTTA
CTCTTGAGAA CTTTGCCAAG AAT**GTAAGA**G CCCTTCCTTA AAACCATGTG
CCACTCTTGA AAATGTCAAC TGTTAGATCC TTTCTGTTTC TGTGTCTGCC
CCATGCACAT TTCATTCTGT TTACTCCTT

Exon I.10

AGA AGAAGAAAAA
GCCATGGCCA TCCCACACAG AGCCAGCTGA GTATTTTACA TGAAAAACAA
AACTGGGGAC TTTTTGAGGT TCTGTCTATA TCACAAAGGG TGAATCACAC
TGGATGGAGA AAGAGACTTA CCTGAGTTAA CAGCCATGCT TCTTCATGAA
GGAGCAATAT GGCCAAGTTG TTGAAACCAA CCATTCCATC TGACACTGTT
TTCAGGGTGA ATCAAACAGC CTTTACTACT ATGGCTAATT GTCTGACTAT
TT**TCCTAG**GT TCCTTACAGG TACTTCCAGC CATTTGGCTT TGGGCCCCGG
GCCTGTGCGG GAAAGTATAT CGCCATGGTC ATGATGAAGG TCACTCTGGT
CATACTTCTG AGACGCTTCC AAGTGCAGAC ACCGCAAGAC CGGTGTGTTG
AAAAGATGCA GAAGAAAAAT GATTTATCCT TGCACCCGGA TGAGACCAGC

Figure 6-2 continued.

GGCCTGCTGG AAATGATTTT CATCCCAAGA AATTCAGACA AGTGTTTCAC
TAAATAAAAT TGGTCAGTCC CTGCCCTGGA CCAGTTCTCA ACAGTATTCC
ACATGGAAAC CACCCATCTT TGCTAGCTAA TCCTCCTCAC ATGAACATTC
TGTGGCCTAT TGTGTTTTAT CAGCTTACCT CCTCTGTGTT ATCAGCATAC
CAGATGCACT GTTCTCCTAA GCATATTCAA GCCAGAAACC AGACTGCAAA
GAACATATGG AGGCCAAGAG TTTGTGCAAG AAACTATAGC CTTAAAGGAC
CATTTCCACA AAACAGGTTT TGGAAAAATG GAGACATCAA CAAACTCATT
CTCATCTCTT CTCTGTTCTA CTGTGAGAAG GGAACCTTTT AATGTCTGGG
GCAGAGGCAC TCAAGTTGAT TAGAAAGGTC CAGCTAACAT CTGGATACCT
ATGGCCAAAC ATACATGGTA ATTTGATTTG GGGTTTGGTG GGATTTGGGG
ACTACAACAT CCGAAGCCTT GAAGAAATGC TTACAAATTC AGCATGTGAC
TTTTTCTATA AATTATATTC AATTAACACT TATTTATTCA CATGTGATTT
ATCTGCAGCA AAAGTTAAAT CAGAGAATAG ACTTGTGGCT GCCTCAGGGG
AGAGGAAGGG AGTGGGAGGA ATCGGGAGCT TGGGGTTAAT GGATGCAATC
TATTGCTCTT GGAATGGATT TACAATGAGA TCATGCTGTG TAGCATTGAG
AACTATGTCT AGATACTTAC ATCGCAACAC AACAGTGGGA GGAAAAAGTA
TGTATACATG TATGTGTAAC TTGGTCCCCA TGCTGTACAA CGGAAATAAA
TTAATTAATT AATTAAAAAA AAAGTTACAT CAAAGAGCAT CCTTTCCCAA
TGTCTCAATT TATGCCTCAA CCAGACCATA TCTCTGCTAT GGGGGGAAAA
AAATCACAAA ATGTGTTTCA AATTTAAATA AAATTCTTTT TTTATGTTGC
ATTGTTATTC TGCCCCAGGA GTACATTAAA CCAAGAGAAT TCCTTAGTTT
AAAAGCATCT ATGAACTCTT ACCACAGCTC CAATGATTTG GAGGAGGAGA
ATATCTGAAT CGTTGTCTAA TTCTACAACC CACTGGGCTC AAAACTCTAA
GGAGTGCTCC TCATACACCA TTATCCTTCT CCTTCAACAC ACGCCCTTCA
CTTTCCATGA CCCCAAGAAC AACAGATCAG CAGAGAACAT GGCCAGGGTG
GACCCCTGAA ATCACTTTTT ATCCTAACTC AGATTTAGAT TTAATAGTTA
CCTTCGAGAT TTAATAATTT TATAGTTTAT TGAATCACTA TATGTAATCT
TGGATAAAAT GTACACCTTA GAGTTAGATC TAGAGAGGGA ATTGAAGAGA
TGACTAAACA TTCCAAATCT ACTTTTCTTT TGTTATATCT ACTGACGGCC
ACTTAAAGAG TGAAAAATTA AACTCACTTA CAAACATGTA AACGTTTGCC
ATTGAGTCAA GCTGAATATC TTTTCATCCT GCCATTTGGC AATCAGGCAA
CACTGAGTGT AAAATGAGAG TGTTATCTCC TGAGAGTGGA AGCTCTTTTC
TTTCCTTTGG AACCTATGCC CATCCCTCAT TCTTACTGTC AATATCAACA
CTCAGTCAAC ACTCTTCATT TATATTCTAA CTGATCCAGA CACCAAGTAA
AAGGTAAGCA GGTATCCTAA GAATTCAGGA TGGGCGTTAG TAAAGAAGGA
AATAGTCGTA CTTTCTGTAC ACCTGAAACT AATAAAATAT TGTAAATCAG
TACTACTCCA ATTTTTACAA AGTA

Figure 6-2 -- continued.

Exon III.2

CTAGAGTACC AGCATCATCT GAGGCAACAG GAGTCCTAAA TGTATATTTT
GGGGCATTGT CTAACTTTTC TGCCCCTCTG ATTT**CCACAG** GATTTTATAT
TGCTTCCCCT GAGATCAAGC AAAGCAAGAT GGTTTTGGAA ATGCTGAACC
CAATGAACAT CAGCAGCATG GTGTCTGAAG CTGTCCTTTT TGGCAGCATT
GCAATCCTGC TGCTCATTGG CTTACTTCTC TGGGTTTGGA ATTATGAGGA
CACATCCTCA ATACCAG**GTA AGT**CAGCCAT TTATTTCTAT ATCTAAGGAG
ATTGTTTTCT TGGGCTTTTG ATCCATAAGG GTAAAGGAAA TTCGTCAAAA
GGAAAAAGAG CAAATCTGGG GAGATGGTTA AAGGGCACGG CATATTAGAA
AATGAAGACT CAGGCAAACA ATTTTAGCCA ACTGTCAGCA CATGTCTAAT
GAAAACATTA AAACTATCCT AAATAATCCC TGCCAGCCTA TACTATGCTT
TGGTAAAATA GACTTGG

Exon III.3

AGAGCCC CATGTTAACT TGTGTGGTTC TTTTAATCAC
TGTGAATGTG TGTGATTCAC CTATATACAT CAGAAGATAT AAGTGGACAA
TCAGATTTTA AAAAAATAAG AACAGCAATA ACTTAAAGCA ATTGCTTTTC
TTGATGTTGC TGAAATACTG ACTGTGATCT AATGACTT**CC CTAG**GTCCTG
GCTATTTTCT GGGAATTGGG CCCCTCATTT CCCACTTCAG GTTCCTCTGG
ATGGGGATTG GCAGTGCCTG CAACTACTAC AACAAAATGT ATGGAGAATT
CATGAGGGTC TGGATAGGTG GAGAGGAAAC ACTCATTATT AGCAA**GTAAG**
TCTGTTAATA ATTGGAGACA CGTTTTTAAA ATCAAGGCTG GGAAATTGCC
TAATAAAAAA CAAACTTATT TTGATAGCTT GCCATCTTTG TTTCCAAAGC
TATGTGTTTA CTACTTGAAT AAAGATGAGT GATTCCCAGG TAAAAAACTA
AGACAAAT

Table 6-1. Genomic Exons Encoding Porcine Cytochrome P450 Aromatase.

	5' Intron-Exon Junction	Exon Size	3' Exon-Intron Junction	Intron Size
Exon I.1A		>116 bp	GACAG	
				0.2 kb*
Exon I.1B		>189 bp	AATGG	
				4.1 kb*
Exon I.2	GACTT	183 bp**	ACCAG	
				2.0 kb*
Exon I.3	GTCCT	151 bp**	AGCAA	
				>6.5 kb
Exon I.4	TGCAG	GTCCT	155 bp	GAAAG GTATT
				4.0 kb
Exon I.5	TTCAG	CTCTG	177 bp	GGATG GTATG
				2.4 kb
Exon I.6	TCCAG	AAAAG	115 bp	TCTGT GTAAG
				2.3 kb
Exon I.7	TGCAG	AAAGG	115 bp	CTGAG GTATG
				0.6 kb
Exon I.8	CTCAG	AAACG	163 bp	TGTTG GTAAG
				2.5 kb
Exon I.9	GACAG	GTGAA	242 bp	AGAAT GTAAG
				1.0 kb
Exon I.10	CCTAG	GTTCC	1198 bp	
Exon III.2	CACAG	GATTT	177 bp	ACCAG GTAAG
				6.0 kb
Exon III.3	CCTAG	GTCCT	151 bp	AGCAA GTAAG
				8.6 kb
Exon IV.4	TGCAG	GTCCT	155 bp	GAAAG GTATT

* Intron sizes determined by PCR using pairs of primers specific for type I porcine cytochrome P450 aromatase and pig genomic DNA as template.

** Exon sizes determined by nucleotide sequence comparisons with the 33F cDNA clone.
AG----- GT : conserved DNA sequences for RNA splicing.

Figure 6-3. Comparisons of genomic and cDNA sequences for porcine cytochrome P450 aromatase isoforms.

Nucleotide sequences of three porcine aromatase cDNAs (#2, the 33F clone; #3, the A10 clone; #4, the P2 clone, respectively; see Chapter 5) were compared with exon sequences of genomic clones (#1', type III; #1, type I porcine cytochrome P450 aromatase) using the PILEUP command of the GCG program. DNA sequence of exons 2 and 3 derived from the type III porcine aromatase gene are shown by bolded, underlined letters. Identical nucleotides except for stop codons (**TAA**) are marked by dots and nucleotide gaps are indicated by dashes (-). The translational initiation codon (**ATG**) is shown for the genomic sequence. The first nucleotide of each exon is indicated by the exon number. The deleted nucleotide sequence corresponding to exons 4 to 6 of the human aromatase gene (#3, the A10 clone), the deletion of six nucleotides (#1', type I aromatase), and the insertion of four nucleotides in the P2 clone are each highlighted by shadowed dashes (▪).

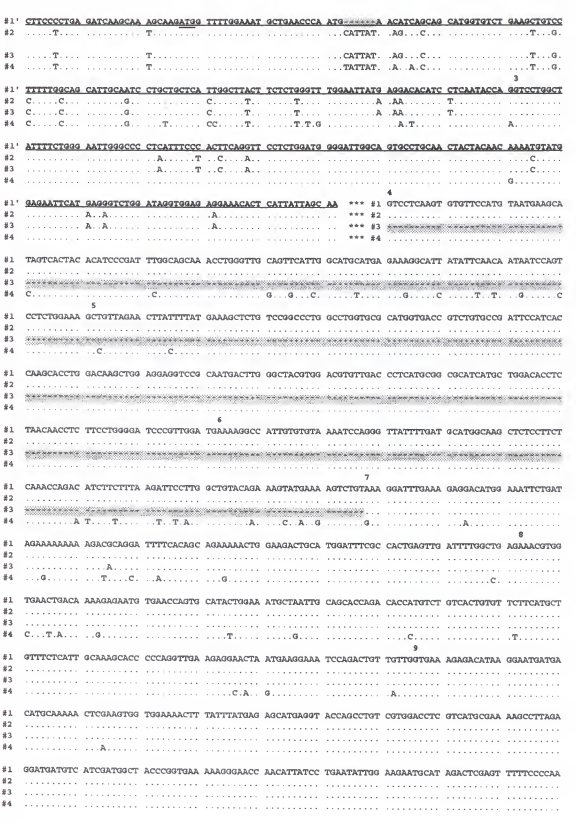
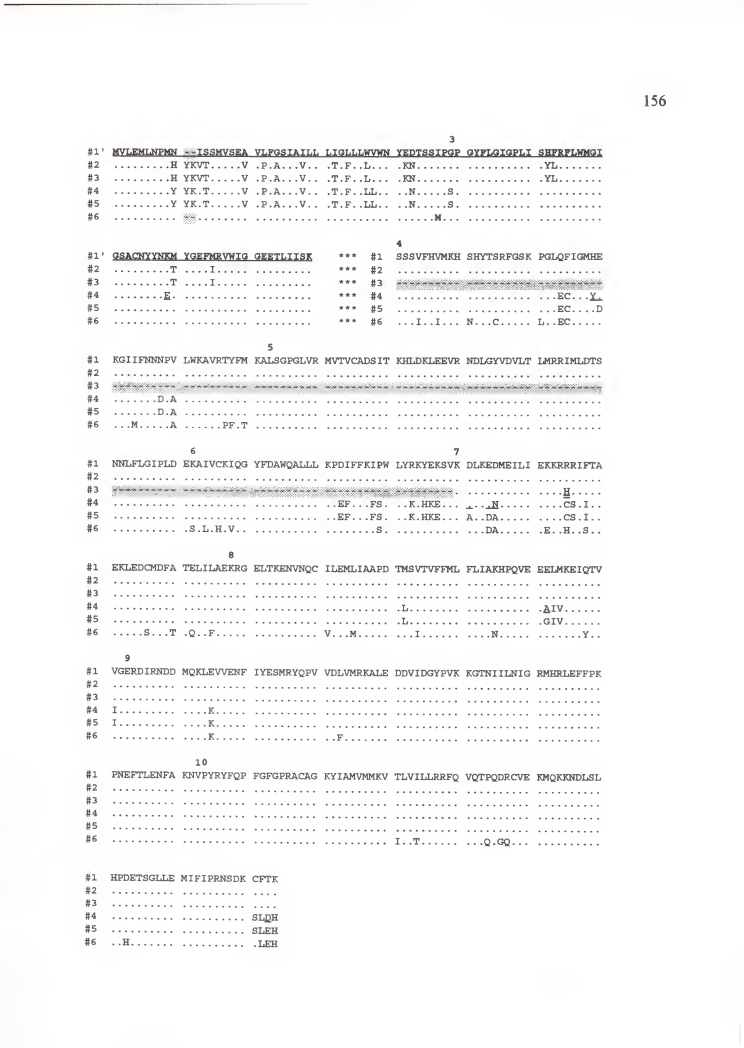


Figure 6-3 -- continued.

```
                                             10
#1  GCCCAATGAA TTTACTCTTG AGAACTTTGC CAAGAATGTT CCTTACAGGT ACTTCCAGCC ATTTGGCTTT GGGCCCCGGG CCTGTGCGGG
#2  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#3  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#4  .......... .......... .......... .......... .......... .......... .......... .......... ..........

#1  AAAGTATATC GCCATGGTCA TGATGAAGGT CACTCTGGTC ATACTTCTGA GACGCTTCCA AGTGCAGACA CCGCAAGACC GGTGTGTTGA
#2  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#3  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#4  .......... .......... .......... .......... .......... .......... .......... .......... ..........

#1  AAAGATGCAG AAGAAAAATG ATTTATCCTT GCACCCGGAT GAGACCAGCG GCCTGCTGGA AATGATTTTC ATCCCAAGAA ATTCAGACAA
#2  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#3  .......... .......... .......... .......... .......... .......... .......... .......... ..........
#4  .......... .......... .......... .......... .......... .......... .......... .......... ..........

#1  G----TGTTT CACTAAATAA AATTGGTCAG TCCCTGCCCT GGACCAGTTC TCAACAGTAT TC
#2  .......... .......TAA .......... .......... .......... .......... ..
#3  .......... .......TAA .......... .......... .......... .......... ..
#4  .AGCC.CGA. ...TAA.G.G GT........ .......... .......... .......... ..
```

Figure 6-4. Alignment of porcine cytochrome P450 aromatase amino acid sequences.

The amino acid sequences of five different pig aromatase isoforms identified here (#2, from the 33F clone; #3, from the A10 clone; and #4, from the P2 clone, respectively) and by others (Corbin et al. 1995) (#5, from placenta and #6, from ovary, respectively) were aligned with those encoded by genomic clones (#1', type III and #1, type I porcine cytochrome P450 aromatase, respectively) by use of the PILEUP command of the GCG program. Identical amino acids are marked by dots and amino acid gaps are indicated by dashes (-). The first amino acid of each exon is marked by a number. The amino acid sequence corresponding to type III porcine aromatase is shown by bolded, underlined letters. The deleted amino acids of exon 2 of the #1' clone and exons 4 to 6 of the #3 clone are highlighted by shadowed dashes (-). The one amino acid of the #6 clone that differs from all others and of the #3 clone that differs from the #2 clone are indicated by bold (**M**) and double underlined (M) letters, respectively. The eight amino acids of the #4 clone that differs from the #5 clone are identified by single underlined letters.



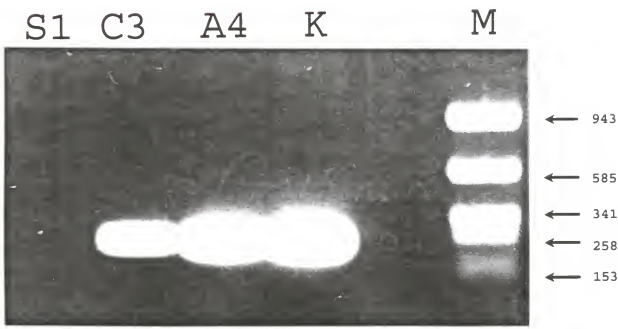


Figure 6-5. PCR amplification of exon 4 and exon-intron boundary sequences from phage genomic clones.

PCR amplifications were carried out for four different phage clones using a pair of primers designed from the intron sequences immediately upstream and downstream of exon 4 of the type I porcine cytochrome P450 aromatase gene (see Figure 6-1). The expected size of the product is 230 bp. The numbers on the right-hand side indicate the sizes (bp) of the DNA standards.

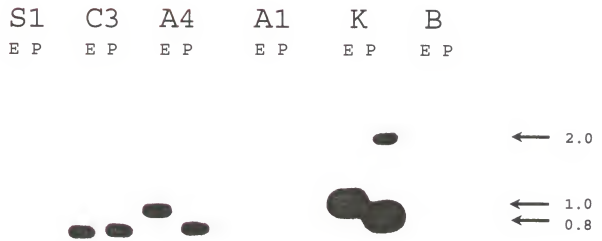


Figure 6-6. Southern blot analysis of phage clones containing porcine cytochrome P450 aromatase DNA fragments.

Genomic clones were digested with restriction enzymes (E: EcoRI and P: PstI, respectively). Southern blot analysis was performed using a radiolabeled aromatase probe containing exon 4 and its boundary sequences (the third EcoRI fragment of the A4 clone, see Figure 6-1). Hybridization to two PstI-digested fragments in the A4 and K clones was due to an internal PstI site in the third EcoRI fragment of the A4 clone. The numbers on the right-hand side indicate the sizes (kb) of DNA fragments that hybridized with the probe.

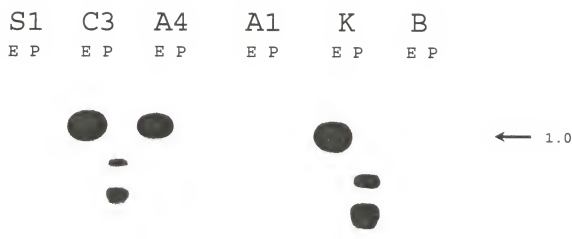


Figure 6-7. Southern blot analysis of six phage clones containing cytochrome P450 aromatase genomic fragments.

Phage clones were digested with restriction enzymes (E: EcoRI and P: PstI, respectively). Southern blot analysis was performed using a radiolabeled porcine aromatase probe containing upstream intron sequence of exon 4 (the first EcoRI fragment of the A4 clone, see Figure 6-1). Hybridizations to three PstI fragments in C3, A4, and K clones were due to internal PstI sites. The numbers on the right-hand side indicate the size (kb) of the DNA fragment that hybridized with the probe.

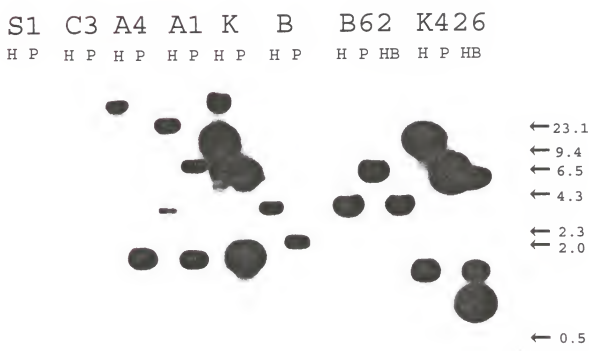


Figure 6-8. Southern blot analysis of phage clones and plasmid subclones containing cytochrome P450 aromatase genomic DNA fragments.

Phage clones were digested with restriction enzymes (H:Hind III and P: PstI, respectively). Two subclones: the B62 subclone containing the genomic fragment lying between the first and third Hind III sites in the B clone and the K426 subclone containing the fragment lying between the second Hind III site and the Sal I site at the end of the K clone, were digested with restriction enzymes (H: Hind III; P: PstI; HB, double digestion with Hind III and BamHI, respectively) (see Figure 6-1). Southern blot analysis was performed using a radiolabeled porcine aromatase cDNA probe containing exons 5, 6, and 7. The numbers on the right-hand side indicate the sizes (kb) of the DNA standards.

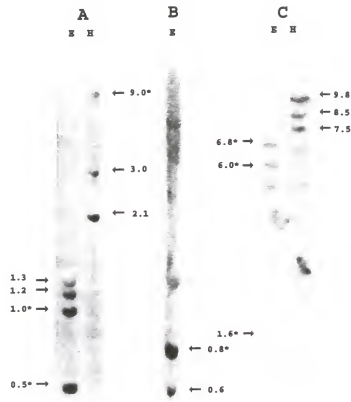


Figure 6-9. Genomic Southern blot analyses.

Genomic DNA extracted and purified from the endometrium of a Day 12 pregnant pig was digested with EcoRI (E) or Hind III (H). Southern blot analyses were performed using a porcine aromatase cDNA probe containing exons 2 and 3 (**A**) or exons 5 to 7 (**C**) of the 33F clone or exon and exon-intron boundary sequences spanning exon 4 (**B**). The numbers on the right and left-hand side indicate the sizes (kb) of the DNA fragments that hybridized with these probes and the numbers with asterisks (*) indicate the expected products, based upon the maps of the individual genomic clones (see Figure 6-1). The 1.6 kb band that was digested with EcoRI enzyme and hybridized with exons 5 to 7 probe is not apparent in the photograph of the autoradiogram.

Figure 6-10. Comparisons of two types of porcine aromatase genomic DNA sequences amplified by PCR from chromosomal DNA.

PCR was performed using a pair of primers designed from exons 7 and 9 of the 33F cDNA sequence (#2 in Figure 6-3 and Materials and Methods) and genomic DNA isolated from endometrium of a Day 30 pregnant pig as template. PCR products (n=8) were cloned and partially sequenced. Two types of DNA sequences (four clones for each type) were identified. The top sequence in each exon is identical to the genomic DNA sequence of type I porcine cytochrome P450 aromatase (#1 in Figure 6-3) and the bottom sequence is identical to that of the P2 clone (#4 in Figure 6-3; type II porcine cytochrome P450 aromatase) in the exon sequence. The amino acid coding sequence of each exon is indicated by capital letters. The nucleotide differences between the two types of clones is indicated by asterisks. The conserved nuclotides for exon-intron splice junctions are indicated by bold, underlined letters.

Exon 7

```
ATAGAAAAAAAAAGACGCAGGATTTTCACAGCAGAAAAACTGGAAGACTGCATGGATTTCGCCACTGAGT
|||||*|||||||||*||||*|||*||||||||||*|||||||||||||||||||||||||||||||||||
ATAGAGAAAAAAAGATGCAGCATTATCACAGCAGAGAAACTGGAAGACTGCATGGATTTCGCCACTGAGT

TGATTTTGGCTGAGgtatggatctggactatccatcaattcccacacaacacagtgtatgattgtttacag
||||||||||*||||************||||||||||*|||||||||*|||||||||*||||||*||
TGATTTTGGCCGAGg..............tatccatcaattccaacacaacatagtgtatgactgtttatag

gtgtctaaaaatt.cactcctgtt.atctgttgcatgtttctgcttaataaagcatctgctctag.tatt
||||||||*|||||*||*|*|||||*||**|*|||||||*|||||||**|*|||*|||||||||*|*||||
gtgtctaacaattcca.ttctgttaat.ggctgcatgtctctgctt..ttaagtatctgctct.gttatt

tctataa..tacccaatgcaactg.ccactattagatatttctgttattactgaccgttaa.aactaaaat
||||*||**||*|||||*|||*|*||||||||||*|**|||*|||**||||||*||||*|||*|||||
tctacaaggt..ccaatgaaacagcccactattagacaccctggtaggactgacctttaacaa.tgaaat

ttaaggtgtcggc
||****|*|***|
tt...ctctgggc
```

Exon 8

```
gaaacat.tcattcacttttccaatgtttcccgcctcotttccattgctcagAAACGTGGTGAACTGAC
|||||*|*|||||||||||||||*|||*||||*||*||*|||||||*||||*|||||||||||*|||*|||
gaaac.tctcattcacttttcaaatatttctcc.cc.cctttcaattgttccagAAACGTGGCGAATTAAC

AAAAGAGAATGTGAACCAGTGCATACTGGAAATGCTAATTGCAGCACCAGACACCATGTCTGTCATCTGTG
|||*|||||||||||||||||||*||||||||||*|||||||||||||||||||*|||||||||||||||
AAAGGAGAATGTGAACCAGTGCATATTGGAAATGCTGATTGCAGCACCAGACACCCTGTCTGTCATCTGTG

TTCTTCATGCTGTTTCTCATTGCAAAGCACCCCCAGGTTGAAGAGG.AACTAATGAAGGAAATCCAGACT
||*|||||||||||||||||||||||||||||||||||||||||||*||*||*|||||||||||||||||
TTTTTCATGCTGTTTCTCATTGCAAAGCACCCCCAGGTTGAAGAGGCAA.TAGTGAAGGAAATCCAGACT

GTTGTTGgtaagaatgtatcaaacaaataatagagtttagaaaacaactccttctgaatatcagcttcag
|||*||||||||||*|||||||||||||||||||*|||||||||||*|*|||||*|||||||*||||*|
GTTATTGgtaagaatttatcaaacaaataatagagctagaaaacaatttcttctaaatatcaacttctg

tgtcctaagaa
||||||||||
tgtcctaagaa
```

Exon 9

```
ccaagactgaactaacctttaactgtactgatctgaaactaggactttatgcccatcacatatagaaaa
|||||||||||||||||||*||*|||||||||||||||||||||||||||||||||||||||||||||||||
ccaagactgaactaacctttacctatactgatctgaaactaggactttatgcccatcacatatagaaaa

taacatgccctcctttcatctgctctgacagGTGAAAAGAGACATAAGGAATGATGACATGCAAAAACTC
||||||||||*|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
taacatgccccctttcatctgctctgacagGTGAAAAGAGACATAAGGAATGATGACATGCAAAAACTC

GAAGTGGTGGAAAAACTTTATTTATGAGAGCATGAGGTACCAGCCTGTCGTGGACCTCGTCATGCGAAAAG
*|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AAAGTGGTGGAAAAACTTTATTTATGAGAGCATGAGGTACCAGCCTGTCGTGGACCTCGTCATGCGAAAAG

CCTTAGAGGATGATGTCATCGATGGCTACCCGGTGAAAAAGGGAACCAACATTATCCTGAATATTGGAAG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCTTAGAGGATGATGTCATCGATGGCTACCCGGTGAAAAAGGGAACCAACATTATCCTGAATATTGGAAG

AATGCATAGACTCGAGTTTTT
|||||||||||||||||||||
AATGCATAGACTCGAGTTTTT
```

```
  1  AAAGGAGTTA GCTGCTGGGG GGAGCTTGGT TGCTGGTGAC AACTGAACCT
 51  ACTTTTGGTG GCATCAACTA TGTCTGTCCT CTCTTTTCTT CCTGCCCCCA
101  TTACTTCTCT TTCTCCTTGT CTTTTATGTT TCCTCTCCTA TTTTTTCTTC
151  TGTCATAGGC TCGCATACAC TACTTCCCTA GCATATGGTT CAAGAGATTT
201  TAAACCTCAT CACAGGAAAT AGGATAAGAT TGTCCTGAGT GAGTCACATT
251  GAATTCAATA GACAACCTCA TGGGAGCTCT GAAATACCTC AATGATGGCC
301  ATGAAACATC TTCTTACTAC AGAAGCTTAC CTTTTGGGGG GAGGTAATTT
351  TGAGCAGGGA AGGGAGTTGG GGATTGTTGT TGTTGTTTTT AATTGGCTTC
                                          CRE
401  AAGGGGAAAA GATTGCCTAA AAAAATTCTG CTGATGAAGT CATTTAATGG
451  CTCCATCTCT GGATTGAGTT TCATTTTCAT GTATTTTGGT AAGAAATTTG
501  GCCCTCAATT GGGAATTCAG TCATTCTACC CACTCAAGGG CAAGATGATA
                                                    SF-1
551  AGTTCTATCA GACCAAGCGT CTAAACGAAC CTGAGACTCT GCCAAGGTCA
601  GAAATGCTGC ATTTCAAGCC AGAGATTTCT CTTGGGCTTA CTTGTTTTGA
651  CTTGTAACTA CAAATATGTC TTGTCTAAGT GTCCAATCAC ATTGTAAAAC
701  AAAATGCCCA TCTCTAGAGT ACCAGCATCA TCTGAGGCAA CAGGAGTCCT
751  AAATGTATAT TTTGGGGCAT TGTCTAACTT TTCTGCCCCT CTGATTTCCA
               ▼
801  CAGGATTTTA TATTGCTTCC CCTGAGATCA AGCAAAGCAA GATGGTTTTG
851  GAAATGCTGA ACC
```

Figure 6-11. DNA sequence upstream of exon 2 of the type III (ovary) porcine cytochrome P450 aromatase chromosomal gene.

The translational initiation codon and putative TATA Box sequence are shown by bolded, underlined letters. The DNA sequences for the consensus CRE and SF-1 binding sites are indicated. The position of the presumptive exon-intron splice junction utilized for human placental aromatase transcripts is marked by a black arrow (▾).

Discussion

In the present study, we provide the first direct evidence for the presence of multiple chromosomal genes encoding isoforms of cytochrome P450 aromatase. Based upon restriction enzyme maps, exon and exon-intron boundary sequences, and comparisons of nucleotide and/or encoded protein sequences, we have identified at least four aromatase genes. Four phages (A1, A4, B, and K clones) collectively encompass exons 4-10 of the type I porcine aromatase gene and encode the 33F clone-type aromatase mRNA/cDNA, which is the major aromatase transcript expressed in pig embryos. The two 5' untranslated exons (E1A and E1B), which are alternatively utilized in embryonic aromatase transcripts (Chapter 4), as well as the first coding exon were localized on the type I aromatase gene by genomic PCR. The intron size between exons 3 and 4 was estimated to be at least 6.5 kb based upon restriction enzyme mapping but the exact intron size was not able to be determined using PCR procedures. Because the size of intron 3 of the type III porcine aromatase gene is ~8.6 kb (this study) and that of the human aromatase gene is ~9 kb (Means et al., 1989; Harada et al., 1990; Toda et al., 1990), it is assumed that the actual size of intron 3 in the type I gene is 8-9 kb. Thus, it is likely that the entire type I porcine aromatase gene is approximately 30 kb in length, which is slightly smaller than the human aromatase genomic DNA region (~35 kb) spanning the protein coding exons. Deletion of exons 4-6 exactly at exon-intron splice junctions in the A10 cDNA clone which otherwise had an identical nucleotide sequence to that of the 33F clone except for one nucleotide in exon 7 region indicates that the A10 clone type transcript probably is derived from the type I aromatase gene.

The S1 phage clone was shown to contain homologous exons 2 and 3 and the peptide sequences encoded by these exons were identical to that of pig ovarian aromatase as reported by Corbin et al. (1995) except for a single amino acid substitution. As shown in Figure 6-4, alignment of the five known isoforms of porcine aromatase and the genomic sequences in the S1 clone revealed that the ovarian aromatase was the only isoform exhibiting a Methionine (M) instead of Isoleucine (I) at position 47. The codon sequence for this amino acid in the S1 clone as well as the cDNA reported in previous chapters (Figure 6-3) is an "ATA" whereas the amino acid "M" is encoded by ATG, suggesting only a single nucleotide difference (A→G) between the S1 clone and the ovarian aromatase cDNA sequence (which is currently not published or available). In addition, a two amino acid deletion found in ovarian aromatase is accounted for by the sequence of the S1 clone. Therefore, it is believed that the S1 clone contains part of the gene, designated as the type III porcine aromatase gene, responsible for production of ovarian aromatase. The 5' flanking region of exon 2 is known to constitute the major aromatase gene promoter utilized in human ovary and corpus luteum (Means et al., 1991; Harada et al., 1993) and several 5' untranslated exons each with their own promoter have been identified upstream of exon 2 in the single human aromatase chromosomal gene. However, it is not yet clear whether there are additional 5' untranslated exon(s) in the type III porcine aromatase gene. In contrast to the S1 clone, the C3 clone contains both ovarian and embryonic aromatase sequences in exons 3 and 4, respectively. Since none of the currently isolated cDNA clones contains this arrangement of exon sequences, it is unclear whether this clone represents a functional gene.

A partial genomic fragment spanning exons 7-9 and including intron-exon boundary

sequences for the type II porcine aromatase gene was obtained by genomic PCR and subcloning. Previously, we demonstrated that the type II aromatase transcript (P2 clone type) was the major mRNA form in pig placenta and endometrium at midpregnancy (Day 60). An additional placental aromatase cDNA clone was isolated from term placenta (Corbin et al., 1995) and eight amino acid differences were found between the two placental aromatase proteins encoded by the P2 and Corbin et al. cDNA clones, respectively. The type II aromatase gene is thought to encode an "E" at position of 502 instead of the "D" in the P2 clone, because five other cDNA clones isolated from Day 60 placenta and endometrium encode the former residue at this position (Chapter 4). Thus, only seven amino acid differences were found between the two placental aromatase protein sequences. There was one amino acid difference in the exon 8 region between the two placental sequences, however, the exon 8 nucleotide sequence of the type II aromatase gene obtained by PCR was identical to that of the P2 clone. In this regard, only a single nucleotide change in the codon (G<u>C</u>A) for the amino acid, "A", in the P2 clone results in the codon (G<u>G</u>A) for the amino acid, "G", in the term placental aromatase sequence. We did not find in any of the clones, obtained by PCR, the corresponding codon for "G". Therefore, we suggest that the two placental aromatase isoforms are actually the same protein and encoded for the type II aromatase gene.

The results from extensive Southern blot analyses of the six phage clones ruled out the presence of additional alternatively utilized exons, which might have contributed to the synthesis of multiple isoforms of porcine aromatase from one gene. Different patterns of hybridization with the probe containing exon 4 and its boundary sequence to the C3 clone

compared to the A4 and K clones (Figure 6-6) suggests that the C3 clone is not derived from the type I aromatase gene. However, the hybridization patterns for EcoRI and PstI fragments using a distinct probe were very similar to each other (Figure 6-7). Since the nucleotide sequence of the 33F clone has high homology with other isoforms of aromatase, we ruled out the possibility that this probe preferentially hybridized with only one type of isoform gene. In addition, the finding of distinct genomic clones encoding the exons 7-9 region for either the placental or embryo type genes also supports the conclusion that these developmentally expressed aromatase isoforms are derived from two separate genes. It was not possible to estimate the number of aromatase genes from Southern blot analysis, however, the results did provide additional evidence for the presence of more than one aromatase chromosomal gene. We therefore, speculate that there exists at least three different aromatase genes in the swine genome; designated herein as type I, type II, and type III genes, respectively. Because the restriction map of each of the phage clones, where overlapping, are similar and because intron as well as exon sequences appear to be highly conserved among these genes, it is likely that these arose from gene duplication events as is the case for the multiple, 3β-hydroxysteroid dehydrogenase (Labrie et al., 1994; Payne et al., 1995) and steroid 11β-hydroxylase (Mornet et al., 1989) genes.

Multiple genes for the 3β-HSD, P-$450_{11\beta}$, and steroid 21-hydroxylase isoforms are known to be closely linked on the same chromosomes, respectively, whereas the genes for the two isoforms of 17β-HSD are located on different chromosomes. It is interesting to speculate whether the occurrence of multiple aromatase chromosomal genes is unique for the pig or occurs in other species as well. Although the existence of a single aromatase

gene in the human is the consensus (Means et al., 1989; Harada et al., 1990; Toda et al., 1990), one cannot rule out the possibility of the presence of multiple very closely related aromatase genes that would be difficult to resolve based on limited restriction endonuclease mapping. Moreover, it is difficult to draw precise conclusions as to the exact copy numbers of genes from the result of genomic Southern blots. The demonstration of the existence of multiple genes encoding isoforms of 3β-HSD in mouse, rat and human suggests the possibility that the presence of isoform-encoding aromatase genes may occur in other species, as well.

It has been reported that the nucleotide sequence of the ovarian promoter (exon 2 plus 5' flanking region) is highly conserved for the several species where identified (Fitzpatrick and Richards, 1994; Hinshelwood et al., 1995; Michaels et al., 1995). Consensus CRE and SF-1 sites, which are found in the human and rat ovarian aromatase promoters (Fitzpatrick and Richards, 1994; Michaels et al., 1995) and in the promoters of all examined steroidogenic enzyme genes (Omura and Morohashi, 1995; Parker and Schimmer, 1995), are found in the DNA sequence upstream of the putative exon 2 of the pig ovarian (type III gene) aromatase gene. It was previously suggested that the ovarian promoter was the primordial aromatase gene promoter (Simpson et al., 1994), because no additional 5'-untranslated exon has been found in the rat and chicken genes and the ovarian promoters were found to be highly conserved for several species. It is of interest to examine the similarity of the 5' upstream sequences of exon 2 in the type I, type II and type III porcine aromatase genes, with the ovarian promoters of other species.

We can only speculate as to the functional and physiological significance of multiple

aromatase isoform-encoding genes and how these genes are regulated in a developmental and tissue-specific fashion. However, one can predict that each enzyme isoform may have different activities for different substrates and/or catalyze synthesis of different products. Although, it is possible that isoforms may have totally unique activities, this remains to be determined. In addition, one can speculate that tissue- and/or developmental specific transcription factor(s) may selectively regulate the expression of specific isoform genes. In this regard, there may be embryo-specific transcriptional factor(s), which by interacting with a specific DNA sequence(s) upstream of the promoters (E1A and E1B) of the type I (embryo aromatase) gene may subserve the temporal expression of porcine embryo aromatase during the periimplantation period. There is as yet no information concerning the possibility that embryo-specific aromatase genes exist in other species. Characterization of the complete gene structures and localization of these multiple genes to a chromosomal region(s) in the pig genom is necessary before the molecular mechanisms controlling their expression can be totally defined. The data presented in this chapter provide the necessary foundation for the eventual understanding of the mechanisms involved in the embryonic and tissue-specific gene expression of cytochrome P450 aromatase and related enzyme isoforms.

CHAPTER 7
SUMMARY AND CONCLUSIONS

To clarify further the nature and mechanisms underlying, interactions between the embryo and maternal uterus during the periimplantation period, studies on the molecular mechanisms of estrogen production by porcine and equine embryos were undertaken. First, the expression of the cytochrome P450 aromatase gene in elongating porcine blastocysts and its temporal expression relationship with uterine luminal IGF concentrations was elucidated as described in Chapter 3. To enable the generation of a homologous aromatase cRNA probe for analysis of conceptus tissue mRNA abundance and to obtain a homologous peptide sequence for generation of monospecific antiserum, a genomic DNA fragment (pAROB9-1) containing exon 9 and exon-intron boundary sequences was obtained by hybridization-screening of a porcine genomic library and subsequent subcloning into a plasmid vector. Using the radiolabeled pAROB9-1 cRNA probe, a major aromatase mRNA transcript of approximately 2.7 kb in size in porcine conceptuses was detected by Northern blot analysis. Northern and Dot blot analyses demonstrated a significantly higher steady-state mRNA abundance of aromatase mRNA in porcine conceptuses at Day 12 than at Days 15 and 18. Results from radioimmunoassay (RIA), using monospecific antiserum to porcine aromatase, demonstrated that total conceptus aromatase content increased from Day 10 to Days 11 and 12 and significantly decreased by Days 15 and 18. To investigate the relationship between

uterine luminal IGF content and aromatase mRNA and protein expression in embryos, the concentrations of IGF-I and IGF-II in uterine luminal fluid (ULF) were estimated by RIAs. Maximal concentrations of IGF-I at Day 12 with a significant decrease by Day 15 and increased concentrations of IGF-II by Day 12 with a constant level until Day 18 of pregnancy were found, respectively. Therefore, the transient expression of conceptus aromatase mRNA and protein in elongating pig blastocysts is coincident with their capacity to secrete estrogens and the uterine IGF-I to IGF-II ratio is positively associated with aromatase synthesis in elongating porcine embryos.

In Chapter 4, as a next step towards understanding the molecular mechanism(s) underlying P450 aromatase gene expression in porcine blastocysts, molecular cloning of cytochrome P450 aromatase cDNAs from periimplantation porcine and equine blastocysts was performed. To begin, a cDNA library from Day 12 porcine conceptuses was constructed. Two types of cDNA clones, Pigaro33F (2470bp) and Pigaro34B (2588bp), which encoded identical aromatase proteins of 503 amino acids but differed in size due to the usage of different polyadenylation signals, were isolated by library screening and were sequenced in their entirety. Two 5' untranslated exons (designated E1A and E1B), which did not show any significant homology with each other nor with 5' untranslated exon sequences of aromatase mRNAs in other species, were obtained from Day 12 porcine conceptuses using the 5'-RACE method. These data provided evidence for alternative splicing of multiple 5' untranslated exons as previously demonstrated for the human aromatase gene. In a comparable study of aromatase gene expression in the female reproductive tract, expression of aromatase mRNA was identified in endometrium and placenta during pregnancy

and in ovary during peri-estrus. Aromatase mRNAs were detected in pig endometrial as well as placental tissues of pregnancy using the RT-PCR method. The finding of aromatase gene expression in endometrium had never been reported previously for any species and these results suggested a possible role of aromatase and estrogen(s) in the local modulation of embryonic and feto-placental growth and/or development during pregnancy and after implantation. Results from RT-PCR demonstrated expression of the E1A exon sequence in Day 30 endometrium and placenta but less (in placenta) or no (in endometrium) expression during Day 60 of pregnancy. Failure of PCR amplification using exon 2 and 3 primers, designed from the 33F clone nucleotide sequence, for Day 60 endometrium and placenta indicated the possibility of additional aromatase isoform-encoding mRNA(s) in endometrium and placenta at midpregnancy. To further test the possibility of the presence of a unique 5' untranslated exon for cytochrome P450 aromatase transcripts, in common for blastocysts of other mammalian species, the 5' cDNA sequence for aromatase was cloned from equine embryos using the 5' RACE method and sequenced. The nucleotide sequence of this 5' untranslated exon did not exhibit any homology with the porcine E1A and E1B sequences.

The results described in Chapter 4 demonstrated the following: 1) the Pigaro33F clone containing the E1A 5' untranslated exon and which utilized the upstream polyadenylation site represents the major form of aromatase mRNA expressed in elongating porcine embryos. 2) Porcine aromatase mRNAs containing either the E1A or E1B exon are formed by alternative splicing mechanisms in pig blastocysts. 3) Pig endometrial as well as placental tissues express aromatase mRNAs during pregnancy; albeit at quite different steady-state levels. 4) Aromatase mRNA containing the E1A exon is expressed in

endometrium and placenta during early pregnancy (Day 30). However, there may be another type(s) of aromatase mRNA containing a third, 5' untranslated sequence expressed in these tissues at Day 60 of pregnancy. and 5) Identification of an aromatase 5' untranslated DNA sequence from horse embryos, which was different from E1A and E1B exons of the pig, suggests that species-specific 5' untranslated sequences are used in embryo aromatase mRNA synthesis and expression.

As a continuation of the studies described in Chapter 4, experiments were carried out to clone the putative aromatase isoform-encoding mRNA(s) favored in endometrium and placenta at midpregnancy. The placental P2 prototype clone encoded a full-length aromatase protein but differed in seventy-seven nucleotides sequence from the embryo cDNA sequence. The same approach was used to clone full-length cDNAs from Day 60 endometrium, Day 30 endometrium and placenta, Day 12 blastocysts, and Day 0 ovary tissues. A variant aromatase cDNA clone (designated A10 clone) was isolated from Day 12 pig blastocysts and was sequenced. This clone was found to be deleted in exons 4-6 (at the exact exon-intron splice junctions of the human aromatase gene), and contained an identical nucleotide sequence to that of the 33F clone except for one nucleotide change in exon 7, and maintained an open reading frame for a 354 amino acid, aromatase-related protein. Results from analyses of restriction endonuclease digestions of amplified PCR products from each tissue and from DNA sequencing of a total of forty-four cDNA clones isolated from these tissues, convincingly demonstrated that the P2 clone-type transcript was the major form in endometrium and placenta at midpregnancy, whereas the 33F clone -type aromatase transcript was the major form in periimplantation embryos and endometrium and placenta during early

(post-implantation) pregnancy. RT-PCR analyses demonstrated that the A10 mRNA was also expressed in pig blastocysts. Lastly, it was indirectly indicated, by RT-PCR analysis, that the P2 mRNA transcript likely contains yet another type of 5' untranslated exon sequence distinct from the E1A and E1B exon sequences.

The results from this Chapter demonstrated: 1) the presence of isoforms of porcine aromatase encoded by distinct mRNAs which are developmentally expressed in blastocysts vs. endometrium and placenta during pregnancy, and 2) the presence of an additional aromatase-related protein-encoding mRNA highly expressed in Day 12 porcine blastocysts, and which may be responsible for the synthesis of unknown estrogen derivatives.

To continue the examination of the molecular mechanisms responsible for the formation of multiple isoforms of porcine cytochrome P450 aromatase and for the temporal expression of the 33F aromatase gene in blastocysts, screening of a porcine genomic library was performed to obtain aromatase chromosomal DNA fragments. These genomic DNA fragments were characterized in detail. Six phage clones, containing the genomic DNA fragments of four different porcine aromatase genes, were isolated and characterized. DNA sequencing of each exon, exon-intron boundary region (about 7,000 bp of total sequence), and the 5' upstream region for exon 2 (800bp) of the type II aromatase gene was performed.

Four types of porcine cytochrome P450 aromatase genes were classified by comparisons of DNA sequences between genomic clones and aromatase cDNA clones. The type I porcine aromatase gene was partially contained within four different phage clones (A1, A4, B, and K clones) that span exons 4-10, and represent the embryonic aromatase (33F

clone type) gene. The type I gene is comprised of 9 coding exons and two mutually exclusive , 5'- untranslated exons (E1A and E1B) and is approximately 30 kb in size. The type II aromatase gene was identified by DNA sequencing of PCR-amplified genomic DNA encoding the exons 7-9 region of the P2 aromatase mRNA. A phage clone (S1 clone) contained the nucleotide sequence potentially encoding the deduced amino acid sequence of ovarian aromatase in exon 2 and 3. An additional phage clone (C3 clone) encoded exons 3 and 4 of ovarian and embryonic aromatase, respectively; however, it is not clear whether this represents yet another type of aromatase gene. Exhaustive Southern blot analyses of the six phage clones, using different porcine aromatase cDNA probes, demonstrated the absence of alternatively used exons that might be involved in the formation of multiple isoform-encoding mRNAs from the same gene. Collectively, results from genomic Southern blot analyses and genomic PCR analysis demonstrated the existence of multiple copies of closely related porcine cytochrome P450 aromatase genes, a finding which has not been reported previously for any species.

It is likely that there exists 1) at least three distinct porcine aromatase genes, which are highly similar in their genomic structure and DNA sequence, in the pig genome, and 2) an embryo-specific gene encoding the 33F and A10 aromatase transcripts is transiently and highly expressed in porcine blastocysts during the periimplantation period and in endometrium and placenta only during early pregnancy.

In conclusion, multiple chromosomal genes encoding multiple, diverse porcine cytochrome P450 aromatase mRNAs and protein isoforms are developmentally expressed in embryos, endometrium and placenta. As such, this is represents a totally novel and

unique mechanism for generating developmental and tissue-specific expression of cytochrome P450 aromatase and its steroidal and possible non-steroidal products and suggests the complexity of regulation of aromatase gene expression (and therefore, estrogen biosynthesis) in embryonic and female reproductive tissues and processes.

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BIOGRAPHICAL SKETCH

Inho Choi was born in Kimpo, Korea, on July 29, 1964. He was educated in Seoul, Korea, prior to coming to the United States. He earned his bachelor degree in February 1988 in the Animal Science department at Korea University. He worked towards a masters degree in Animal Science under the guidance of Dr. Yong-Suk Son. He earned a degree in the field of ruminant nutrition with the thesis title "A Study of Adaptative Responses of Sheep to High-Nitrate Feeding", in February 1990. He then joined Dr. Frank A. Simmen's laboratory in May 1991 as a Ph.D student in the department of Dairy and Poultry Sciences and the Interdisciplinary Concentration in Animal Molecular and Cell Biology at the University of Florida and completed the requirements for the degree of Doctor of Philosophy in May 1996. He will pursue postdoctoral research with Dr. Benita S. Katzenellenbogen in the Molecular and Integrative Physiology Department at the University of Illinois in Urbana, Illinois, beginning May 1996.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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